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(54) Title: THERAPEUTIC BLOCKADE OF ICER SYNTHESIS TO PREVENT ICER–MEDIATED INHIBITION OF IMMUNE CELL ACTIVITY

(57) Abstract

The present invention relates to reagents and methods for modulating immune cell activity using agents which regulate the activity of ICER.

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THERAPEUTIC BLOCKADE OF ICER SYNTHESIS TO PREVENT ICER-MEDIATED INHIBITION OF IMMUNE CELL ACTIVITY

Background of the Invention

Many human afflictions, including cancer and diseases caused by infectious organisms, result from the ability of their causative agents to escape destruction by immune cells. In particular, recent studies have indicated that tumor rejection and the destruction of infectious organisms by the immune system are critically dependent upon the occurrence of adequate antigen-presenting cell (APC) function and T cell restimulation within tumor deposits. Many tumors and infectious pathogens probably escape immune recognition and rejection through local inhibition of APC and lymphocyte function.

For example, cancer cells may induce the production of prostaglandin E2 (PGE2) in neighboring normal host cells such as macrophages. Allev, D.G. et al., Scand. J. Immunol. 39:31-38, 1994. PGE2 inhibits the prolifieration of T cells, thereby reducing the ability of the host immune system to destroy the cancer cells.

Tumor cells and infectious agents may also produce other substances which directly or indirectly reduce the activity of the host's immune response. Accordingly, there is a need for therapeutic strategies which prevent or reduce downregulation of the host immune response.

The present invention relates to strategies for preventing or reducing downregulation of the immune response in individuals suffering from afflictions in which downregulation of the immune response contributes to the progress of the disease. Such afflictions include cancer or infection with a pathogen.

Summary of the Invention

One aspect of the present invention is an agent which decreases the level of ICER expression for use in increasing the activity of an immune cell. In some embodiments, the agent comprises a nucleic acid. In some embodiments, the nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA. In other embodiments, the nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

Another aspect of the present invention is the use of an agent which decreases the level of ICER expression in the preparation of a medicament for increasing the activity of an immune cell. In some embodiments, the agent comprises a nucleic acid.

In some embodiments, the nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA. In other embodiments, the nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA. In some embodiments, the medicament is for the treatment of cancer. In other embodiments, the medicament is for the treatment of infection with a pathogenic organism. In some embodiments, the immune cell is selected from the group consisting of T cells, B cells, NK cells, monocytes, dendritic cells and antigen presenting cells. In other embodiments, the immune cell is a T cell.

Another aspect of the present invention is a ribozyme comprising at least one catalytic sequence having endonuclease activity and at least one targetting sequence, wherein the at least one targetting sequence is complementary to a sequence present in at least one isoform of ICER mRNA.

Another aspect of the present invention is an immune cell into which an agent capable of decreasing the expression of ICER has been introduced. In some embodiments, the agent comprises a nucleic acid. In some embodiments, the nucleic acid comprises a ribozyme comprising at least one catalytic sequence having endonuclease activity and at least one targetting sequence, wherein the at least one targetting sequence is complementary to a sequence present in at least one isoform of ICER mRNA therein. In other embodiments, the nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA. In other embodiments, the immune cell is selected from the group consisting of T cells, B cells, NK cells, monocytes, dendritic cells and antigen presenting cells. In some embodiments, the immune cell is a T cell.

Another aspect of the present invention is a method for increasing the activity of an immune cell comprising decreasing the level of ICER expression in said immune cell. In some embodiments, the level of ICER expression is decreased by introducing a nucleic acid which inhibits ICER expression into the immune cell. In some embodiments, the nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA. In other embodiments, the nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA. In some embodiments, the immune cell is selected from the group consisting of T cells, B cells, NK cells, monocytes, dendritic cells and antigen presenting cells.

Another aspect of the present invention is a method of increasing the activity of immune cells in an individual comprising the steps of removing immune cells from the individual, introducing an agent which inhibits ICER expression into the immune cells, and reintroducing the immune cells into the individual. In some embodiments, the agent comprises a nucleic acid. In some embodiments, the nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

In some embodiments, the nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

In other embodiments, the immune cell is selected from the group consisting of T cells, B cells, NK cells, and antigen presenting cells. In further embodiments, the immune cell is a T cell. In some embodiments, the individual suffers from a condition which reduces immune cell activity. In other embodiments, the individual is infected with a pathogenic organism. In other embodiments, the individual suffers from cancer.

Another embodiment of the present invention is a method of increasing the activity of immune cells in an individual comprising introducing an agent which inhibits ICER expression into immune cells in the individual. In some embodiments, the agent comprises a nucleic acid. In some embodiments, the nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

In other embodiments, the nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

In some embodiments, the immune cell is selected from the group consisting of T cells, B cells, NK cells, and antigen presenting cells. In other embodiments, the immune cell is a T cell. In some embodiments, the individual suffers from a condition which reduces immune cell activity. In other embodiments, the individual is infected with a pathogenic organism. In further embodiments, the individual suffers form cancer.

Brief Description of the Drawings

Fig. 1 provides the nucleotide sequence of the ICER gene (SEQ ID NO. 1)

Fig. 2 illustrates the structures of the four ICER transcript isoforms.

Fig. 3 illustrates the ICER expression pattern in T cells treated with ionomycin, forskolin, or ionomycin + forskolin.

Fig. 4 illustrates the ICER expression pattern in monocytes treated with forskolin, prednisolone, IL-10, GM-CSF, and ionomycin.

Fig. 5 illustrates the inhibitory effects of forskolin treatment on the proliferation of T cells contacted with a variety of agents which normally induce proliferation.

Fig. 6 illustrates the ICER expression pattern in monocytes whose ionophore-induced differentiation is inhibited by forskolin.

Fig. 7. Cyclic AMP-mediated attenuation of T helper-1 cytokine transcription in human medullary thymocytes correlates with cAMP-mediated induction of the transcriptional repressor ICER. RNA from untreated (U) medullary thymocytes and forskolin (F) or 8-Bromo-cAMP (Br) treated human medullary thymocytes in the absence (lanes 2 and 3) or presence of phorbol ester and ionomycin (PMA + Iono); (lanes 5 and 6) were scored for cytokine expression using RIBOQUANT probes hCKJ (A) and hCK3 (B) in the RNase protection assay. Also shown are the corresponding RNase-protected robes following hybridization with yeast tRNA in the presence (+) (lane 7) or absence (-) (lane 8) of RNase. Templates for the analysis of hL32 and hGAPDH housekeeping genes were included to allow assessments of total RNA levels (Pharmingen). Note that each probe (lane 8) migrates slower than its protected band; this is due to flanking sequences in the probe that are not protected by mRNA. (C) Western immunoblotting using ICER-specific antiserum generated against a peptide encompassing the ICER-specific exon (3b) shows induction of ICER in medullary thymocytes after 3hrs, but not after 12 hrs of forskolin treatment. There is no ICER protein detectable before treatment or in forskolin-treated human cortical thymocytes which corresponds to a lack of detectable ICER mRNA reported previously in cortical thymocytes (36).

Fig. 8. ICER binding and formation of an NFAT/ICER complex with NFAT/AP-I composite DNA-binding sites of the IL-2 promoter. (A) List of NFAT/AP-1 composite sites of IL-2 promoter delineated previously to be essential for IL-2 expression (63) used in EMSA analysis. NFAT and AP-1 (top) denote domains of homology between NFAT/ AP-1 composite sites and consensus sequences for NFAT and AP-1 in human IL-2 promoter, respectively. Numbers on the left correspond to the relative distance of the depicted DNA-binding motifs from TATA box of the IL-2 promoter. (B) Bacterially expressed ICER binds in boiled total bacterial lysate specifically to (-90) (lanes 3 and 4) and (-160) CD28RE

motifs (lanes 7 and 8) and to a limited extent also to the rest of the motifs. The binding of ICER to these motifs is specific since it is recognized by CS4 CREM-specific antiserum (CS4) causing a specific supershift (sICER) while the nonspecific complex (NS) remains unperturbed both in the presence of CS4 (+) and normal rabbit antisera (-). The control CRE consists of an oligonucleotide encompassing 21 bp repeat of HTLV-I LTR (H21CRE) (64). (C) In vitro binding of purified recombinant ICER and NFAT DBD (NFAT) proteins yields NFAT/ICER ternary complex (NF/IC) on CD28RE motif (460) (lane 12) and to lower extent also on NFAT -45 motif (lane 3). (D) ICER and truncated Fos and Jun proteins (AP) form similar complexes (NF /IC versus NF / AP) in the presence of NFAT DBD (NFAT) on the (-160) motif of the IL-2 promoter (lanes 3 and 9), which are recognized by the CS4 anti-CREM (C) (lane 4) and DX anti-Fos (D) (lane 10) or K25 anti-Jun (K) (lane 11) and to a limited extent also by R59 NFAT (R) antisera (lane 12), respectively. Both unlabeled oligonucleotides NFAT (nf) (lane 6 and 13) and AP-1 (ap) (lanes 7 and 14) efficiently compete for complex.

Fig. 9 ICER protein can interact directly with NFAT DBD. Decreasing amounts of NFAT DBD protein retained on Sepharose matrix with equal amounts of GST-linked ICER (GST-ICER, lanes 1, 3, 6) alongside with a negative control represented by equivalent amounts of GST matrix alone (GST, lanes 2, 4, 7) interact specifically in GST-pull down assay. Lane 5 represents the input of NFAT DBD which is equivalent to protein added to GST-ICER beads retained in GST-pull down in lane 3. In contrast, GST-CREB (lane 10) does not interact with comparable amounts of NFAT DBD protein (input, lane 8). Lane 9 represents GST-CREB beads alone. NFAT DBD retained on GST-ICER Sepharose beads was separated by SDS-PAGE and visualized by Western blotting using the NFAT-specific antibody R59.

Fig. 10 ICER binds to a conserved proximal element of the IFNγ promoter homologous to the (-90) motif of IL-2 promoter (A) ICER binds to both human and mouse motifs of the IFNγ promoter (lanes 2 and 9), despite that these motifs fail to bind NFAT DBD (NFAT) (lanes 3 and 10 4 a fashion analogous to the (-90) motif of the IL-2 promoter. No complex formation between ICER and NFAT was detected in EMSA (lanes 4 and 11). ICER bound to the proximal element of the IFNγ promoter is specifically "supershifted" (sICER) by a CS4 CREM-specific antibody (C) (lanes 5 and 12). The mobility of the ICER complex is altered using competition with unlabeled CRE oligonucleotide (cre) (lanes 6 and 13) but unchanged in the presence of unlabeled NFAT oligonucleotide (nf) (lanes 7 and 14). (B) Sequences of human and mouse proximal elements of the IFNγ promoter show significant homology to the (-90) motif of the human IL-2 promoter.

Fig. 11 ICER forms complexes on several NFAT/AP-1 composite sites in GM-CSF, IL-4, and TNF-a promoters. (A) A list of NFAT/AP-1 composite sites previously identified as essential for the expression of GM-CSF (54), IIL-4 (52), and TNF-α (55, 56) promoters used in EMSA analysis. (B) Purified ICER and truncated NFAT DBD proteins form in vitro NFAT/ICER complexes on enhancer core of GM-CSF promoter GM-420 (lane 6), proximal NFAT/AP-1 motif of the IL-4 promoter [IL4(-80)] (lane 12), and the κ3 motif of TNF-α (κTNF-α) (lane 15) with affinities comparable to the CD28RE motif (-160) of the IL-2 promoter (Fig. 8). (C-E) NFAT/AP-1 composite sites in GM-CSF, IL-4 and TNF-α promoters bind to ICER and form ICER containing complexes in whole cell extracts prepared from freshly prepared

human medullary thymocytes. Bacterially expressed, purified ICERII, and ectopically expressed full length NFATI protein in Cos cells, served as controls (lanes 1 and 2, respectively) for evaluation of extracts (lanes 3 -11) prepared from untreated human medullary thymocytes (C) or human medullary thymocytes treated with forskolin (3h) and ionomycin (15') (D), E). ICER and ICER containing complexes were competed by unlabeled oligonucleotides containing CRE motifs (cre) (lanes 4, 7, 10) or NFAT motifs (nf) (lanes 5, 8, 11) (panels C and D) and were immunoreactive with CS4 CREM-specific antisera (C) (panel E, lanes 4, 7, 10), whereas the R59 NFAT-specific antisera (R) (lanes 5, 8, 11) affected the mobility of numerous com l xes, but shows little effect on the binding of ICER (panel E).

Fig. 12 ICER isoform II represses transcription from NFAT/AP-1 activated cytokine promoters stimulated by PMA and ionomycin (P+I) treatment. (A) Promoter-reporter, IL-2-CAT (human interleukin-2 (61) CD28RE (-160 AP-Luc); GM-CSF-CAT (54) (human granulocyte-macrophage colony stimulating factor), TNF-α-Luc (contains the sequence from -614 to +20 of the human tumor necrosis factor-a in pGL2). A control (3xGAL4)-CR-CAT (with three GAL-4 binding sites substituting 21-bp repeats in the HTLV-1 LTR, (64)) and transactivated by GAL4VP16 (73) is not affected in Jurkat cells by isoforms of ICER (ICER II, ICER IIy, ICER 1). (B) Amounts of respective cytokine reporters and ICER expression constructs in transient transfectiom were kept constant (2 μg). Error bars represent standard deviations calculated from three or more experiments.

Fig. 13. Peripheral blood T lymphocytes can induce stable ICER protein upon forskolin (F) or PGE_2 (P) treatment. Immunoprecipitations of total ^{35}S labeled cell lysates using CREM-specific antiserum (C) and normal rabbit serum (N) show accumulation of ICER protein in peripheral blood T lymphocytes after 3, 12, and 18 hrs of forskolin (0.1 mM final) (F3, F12, F18) or PGE_2 (500ng/ml final) treatment (P3, P12, P18). ICER protein is barely detectable in untreated (U) negatively selected T cells (lanes 1 and 2) while clearly detectable 3hrs after the both treatments (lanes 3 and 4, and lanes 9 and 10, respectively) reaching the maximum first in the presence of forskolin after 12 hrs (lanes 5 and 6) and then in the presence of PGE_2 at 18 hrs after the treatment (lanes 13 and 14).

Fig. 14. Forskolin-mediated transcriptional attenuation of cytokine and chemokine expression in IL-12 induced Th1- and IL-4 induced Th2-like human peripheral blood T lymphocytes correlates with induction of potent transcriptional repressor ICER. *In vitro* polarized T cell populations with Th1 or Th2 dominant phenotypes were evaluated by flow cytometry analysis (A). Shown are T lymphocytes (containing more than 95% of $CD3^+$ cells) which were typically obtained after *in vitro* priming and polarization toward the Th1 or Th2 dominant phenotypes. The non-$CD3^+$ cells were $CD14^+$ (1% in Th1 and less than 1% in Th2), $CD19^+$ (1% in Th1 and 2% in Th2) and $CD16^+$ (2% in Th1 and 5 % in Th2). PBMC primed as described were maintained in the medium containing IL-2 until the restimulation. In the experiment shown, the cells were cultured for a total of two weeks prior to restimulation, Th1 and Th2 populations shifted significantly towards the memory phenotype, represented by the CD45RO marker. In the Th1 population 57% of $CD4^+$ cells and 35% of $CD8^+$ cells maintained the same ratio of $CD4^+$ versus $CD8^+$ after polarization as prior to polarization (data not shown). The Th2 dominant culture contained 60% of $CD4^+$ and 14% of $CD8^+$ cells. RNAs from IL-12 induced Th1 and IL-4 inducedTh2 dominant populations were restimulated with PHA either in the absence (-) or presence of forskolin (+) and scored in parallel for human cytokine (B) and chemokine

expression (D) in parallel with ICER mRNA(C and E) using the RNase protection assay. For evaluation of expression of human cytokines (B) a Riboquant set of hCK1 probes (hIL-5, hIL-4, hIL-10, hIL-14, hIL-15, hIL-9, hIL-2, hIL-13, and hIFN-γ) was used while for evalution of expression of human chemokines (D) was used Riboquant set of hCK5 probes (hLtn, hRANTES, hIP10, hMIP1β, hMIP1α, hMCP1, hIL-8, hI-309) (PharMingen). Levels of cytokine or chemokine expression in activated Th1 and Th2 lymphocytes after forskolin treatment inversely correlated with levels of ICER mRNA (C and E). Templates for the analysis of hL32 and hGAPDH housekeeping genes were included to allow assessment of total RNA levels (PharMingen). Note that each probe migrates slower than its protected band; this is due to flanking sequences in the probe that are not protected by mRNA.

Fig. 15. Differential susceptibility of cytokine expression in Th1 and Th2 dominant subsets to cAMP-mediated inhibition after restimulation with either phorbol ester plus ionophore (P+I) or phytohemaglutinin (PHA). Human peripheral blood T lymphocytes primed with OKT-3 and polarized in the presence of IL-12 (Th1) or IL-4 (Th 2) were restimulated for 6 hours with P+I or PHA in the absence or the presence of forskolin. After restimulation RNAs were scored in the RNase protection assay for cytokine and ICER expression using human cytokine Riboquant set of hCK1 probes (hIL-5, hIL-4, hIL-10, hIL-14, hIL-15, hIL-9, hIL-2, hIL-13, and hIFN-) (PharMingen) and JL5 probe (74) respectively. Also shown are the corresponding RNase-protected probes following hybridization with yeast RNA in the presence (lane 13) or absence (lane 14) of RNase. Templates for the analysis of hL32 and hGAPDH housekeeping genes were included to allow assessments of total RNA levels (PharMingen, San Diego, CA). Note that each probe (lane 14) migrates slower than its protected band; this is due to flanking sequences in the probe that are not protected by mRNA.

Fig. 16. ICER-mediated transcriptional attenuation of cytokine and chemokine expression in activated lymphocytes from ICER transgenic mice. Freshly isolated ICER transgenic (+) or control nontransgenic (-) thymocytes were activated with PMA + ionomycin for three hours. RNAs were isolated and analyzed in the RNase protection assay in parallel for cytokine (A) or chemokine production (B) as well as ICER expression (data not shown) using Riboquant sets for mCK1 probes (mIL-4, mIL-5, mIL-10, mIL-13, mIL-15, mIL-9, mIL-2, mIL-6, m INF-γ,) or mCK5 probes (mLtn, mRANTES, mEotaxin, mMIP-1β, mMIP-1α, mMIP-2, mIP-10, mMCP-1, mTCA-3) (Pharmingen), respectively. Also shown are the corresponding RNase-protected probes following hybridization with yeast RNA in the presence (lanes 4) or absence (lanes 5) of RNase. Templates for the analysis of hL32 and hGAPDH housekeeping genes were included to allow assessments of total RNA levels (PharMingen, San Diego, CA). Note that each probe (lane 5) migrates slower than its protected band; this is due to flanking sequences in the probe that are not protected by mRNA.

Fig. 17. A proliferative defect in lymphocytes from ICER transgenic mice. (A) Freshly isolated thymocytes from ICER transgenic or control nontransgenic littermates were isolated, and total ^{35}S-labeled cell lysates were assayed by immunoprecipitation using a CREM specific antiserum (c) along with control normal rabbit serum (n). Proliferation of splenocytes (B) activated for 48 hrs with ConA, PMA plus ionomycin (PMA+ionophore) or by immobilized anti-CD3 monoclonal antibody (2C11) was measured by ^{3}H-thymidine incorporation. (C) Allogeneic

response in mixed lymphocyte reaction of C57BL/6 splenocytes from ICER transgenic or nontransgenic control mice to allogeneic BALB/c splenocytes. Irradiated, unfractionated splenocytes from syngeneic C57BL/6 or allogeneic BALB/c splenocytes were added to cultures containing unfractionated ICER transgenic or nontransgenic C57BL/6 splenocytes. After 48 hours of coincubation thymidine uptake was measured as described. (D) Cell yields from thymus and spleen of transgenic and nontransgenic animals.

Fig. 18. ICER proteins are induced early in response to forskolin in freshly isolated human peripheral blood $CD3^+$ T lymphocytes but only to limited extent in $CD19^+$ B lymphocytes. Immunoprecipitations using CREM-specific antiserum (C) (116) and normal rabbit antiserum (N) show induction of ICER proteins (17-20 kDa) in unseparated lymphocytes after three hours of forskolin (F) treatment (0.1 mM final) (lanes 1 to 4). Different isoforms of ICER proteins are detectable in untreated (U), positively selected, $CD3^+$ T cells (lanes 5 to 8) showing higher background levels in comparison with positively selected $CD19^+$ B cells (lanes 13 to 16) and/or their respective pass-throughs ($CD3^+$PT, $CD19^+$PT) (lanes 9 to 12, and 17 to 20, respectively). Purity of freshly isolated $CD3^+$ T cells and $CD19^+$ B cells was evaluated by subsequent flow cytometry analysis. Background levels of ICER proteins are specific since control immunoprecipitations of untreated Jurkat T cells (lanes 21 and 22) do not yield any detectable ICER protein in agreement with lack of detectable ICER mRNA (see Fig. 22B, lane 1) while after forskolin treatment both ICER mRNA (see Fig. 22B, lane 2) as well as ICER proteins (lanes 23 and 24) are detectable. Similarly, immunoprecipitations of B cells ($CD19^+$) (lanes 13 to 16) as well as its pass-through population ($CD19^+$PT) (lanes 17 to 20) show modest although detectable induction of ICER proteins after forskolin treatment in comparison with untreated cells.

Fig. 19. Forskolin-mediated FasL down-regulation correlates with ICER induction in activated 2B4 T cell hybridoma. A, RNA from phorbol ester (10^5 mg/ml) and ionomycin (1 g/ml) (P+I) activated 2B4 T cell hybridoma treated for indicated time in the presence of forskolin (0.1 mM final) (P+I+F) or absence of forskolin (P+I) or in the presence of forskolin alone (F) were scored in parallel either for ICER expression (A) or for FasL expression (B) using ICER or mAPO3 RIBOQUANT probes in the RNase protection assay. Under these conditions FasL expression as assessed by RNase protection used for evaluation of mRNA of multiple constituents of Fas and TNF pathways in 2B4 T cells (FLICE, FasL, Fas, FADD, FAP, FAF, Fas2L, TNFRp55, TRADD, and RIP) was dramatically altered after the treatments (B). Activated phorbol ester-ionomycin treated 2B4 T cells induce ICER in the presence of forskolin after three hrs (P+I+F) (lane 6) to similar or even higher levels than after forskolin treatment alone (F) (lane 18). There is minimal induction of ICER mRNA detectable after phorbol ester-ionomycin treatment alone (P+I) (lanes 7 to 12). B, Forskolin mediated ICER induction shows inverse correlation with FasL expression. Identical RNAs scored for ICER induction in panel A were subjected to RNase protection using mouse RIBOQUANT probe mAPO3 in panel B. After three hours of combined treatment (P+I+F) minimal amounts of FasL message could be detected (lane 6). Forskolin-mediated attenuation of FasL expression in activated 2B4 cells (panel B; lane 6) tightly correlates with forskolin-mediated induction of the transcriptional repressor ICER (panel A; lane 6) while expression of FasL remains unperturbed in activated 2B4 T cells in the absence of ICER (P+I) (panel B, lane 12). Also shown are the corresponding RNase-protected probes following hybridization with yeast tRNA in the presence (+) (lane 19) or

absence (-) (lane 20) of RNase. Templates for the analysis of mL32 and mGAPDH housekeeping genes were included to allow assessments of total RNA levels (PharMingen, San Diego, CA). Note that each probe (lane 20) migrates slower than its protected band; this is due to flanking sequences in the probe that are not protected by mRNA.

Fig. 20. FasL down-regulation correlates with increased ICER expression in activated human peripheral blood T lymphocytes after forskolin treatment. Purity of negatively selected human peripheral blood T cells (Pan T) was evaluated by flow cytometry analysis. RNAs from freshly prepared human peripheral blood T lymphocytes were treated for three hours with phorbol ester and ionomycin (P+I) either in the presence of forskolin (F) or absence of forskolin (U) and scored in parallel for FasL or ICER mRNAs using RNase protection assay with hAPO3 RIBOQUANT or ICER probes, respectively. Activated human peripheral blood T cells after phorbol ester and ionomycin treatment (P+I) show decreased levels of FasL message in the presence of forskolin (P+I/F) (top, lane 1) accompanied by increased levels of ICER (middle, lane 1). Conversely, in the absence of forskolin (P+I/U), T cells activated by phorbol ester and ionomycin expressed higher levels of FasL message (A, lane 2) accompanied by lower levels of ICER mRNA (middle, lane 2). There is no FasL and very little ICER message present in untreated cells (U) (lane 4). Templates for the analysis of hL32 and hGAPDH housekeeping genes (bottom) were included to allow assessment of total RNA levels (PharMingen).

Fig. 21. Induction of FasL expression in phorbol ester activated human peripheral blood NK lymphocytes is accompanied by cessation of ICER expression. Elutriated peripheral blood lymphocytes were used to isolate $CD56^+$ NK cell population (Miltenyi Biotec, Auburn, CA). Purity of $CD56^+$ NK cell population was evaluated by flow cytometry analysis (see Experimental Procedures). RNAs from either untreated (U), and one (P1) or three hours (P3) of phorbol ester treated cells were scored using RIBOQUANT probe hAPO3 (PharMingen, San Diego, CA) (A) or ICER (B). Also shown are elevated levels of mRNAs for hFAP and hTRADD and the corresponding RNase-protected hAPO3 probes following hybridization with yeast tRNA in the presence (+) (lane 3) or absence (-) (lane 4) of RNase. Templates for the analysis of hL32 and hGAPDH housekeeping genes were included to allow assessments of total RNA levels. Note that each probe (lanes 1 and 2) migrates slower than its protected band (lane 4); this is due to flanking sequences in the probe that are not protected by mRNA.

Fig. 22. Elevated levels of ICER can be detected in human peripheral blood $CD56^+$ NK cells as well as in human NK cell lines NK3.3 and NK92 prior to forskolin treatment. A, Immunoprecipitations using CREM-specific antiserum (C) or normal rabbit antiserum (N) show presence of ICER protein in untreated $CD56^+$ NK cells (U) (lane 2) with moderate increase after three hours of forskolin (F) treatment (lane 4). ICER protein is detectable in untreated $CD56^+$ NK cells prior to forskolin treatment. B, RNAs from human NK cell lines NK 92 and NK 3.3 were scored in RNase protection assay for ICER mRNA using ICER probe prior and after three hours of forskolin treatment (lanes 3 to 6). Both NK3.3 and to a lower extent also NK92 human NK cell lines exhibit elevated levels of ICER mRNA prior to forskolin treatment in contrast to Jurkat T cell line which does not express detectable amount of ICER mRNA prior to forskolin treatment (lane1).

Fig. 23. ICER binding and formation of an NFAT/ICER complex with proximal NFAT motif of the FasL promoter. A, Listed NFAT motifs of FasL promoter delineated previously to be essential for NFAT-driven FasL expression in T lymphocytes (171, 172) were used in electromobility shift assay analysis along with control NFAT/AP-1 composite sites in IL-2 and GM-CSF promoters which can bind ICER directly as well as indirectly (166). NFAT and AP-1 (top) denote domains of homology between NFAT/AP-1 sites and consensus sequences for NFAT in human Fas ligand, and NFAT and AP-1 sites in IL-2, and GM-CSF promoters, respectively. Numbers on the left correspond to the relative distance of the depicted DNA-binding motifs from the transcription initiation site. B, Purified, bacterially expressed ICER binds specifically to proximal NFAT motif of Fas ligand promoter (lanes 1 and 2) and to a limited extent also to the distal NFAT motif (lanes 3 and 4). The binding of ICER to these motifs is specific, since it is recognized by CS4 CREM-antiserum (CS4), causing a specific "supershift" of ICER (sICER). C, *In vitro* binding of purified recombinant ICER and NFAT DBD (NFAT) protein yields NFAT/ICER ternary complex (NF/IC) on proximal NFAT motif of FasL promoter (lane 3) but not on distal one (lane 6). The control NFAT/AP-1 composite sites consists of oligonucleotides encompassing (-160) position of human IL-2 promoter (lanes 7 to 9) and (-420) position of human GM-CSF promoter (lanes 10 to 12) which bind ICER directly and/or yield NFAT/ICER ternary complex (NF/IC) (166). Free, stands for free probe.

Fig. 24. ICER represses transcription from luciferase reporter of human FasL promoter activated by anti-CD3ε antibody (clone 2C11) in 2B4 T cell hybridoma; hFasL (-225) Luc reporter contains proximal NFAT binding site (from -144 to -126); hFasL (-511) Luc reporter contains both proximal (from -144 to -126) and distal (from -283 to -263) NFAT sites (panel A). Neither mock transfection nor control pGl3 empty Luc reporter (pGl3emptyLuc) transfection alone show significant luciferase activity in 2C11-stimulated 2B4 T cells (panel B). Different isoforms of human ICER (ICERII, ICERIIγ ICERI, and ICERIγ) have differential inhibitory effect depending on used FasL reporter (panel B). Amounts of respective FasL reporters and ICER expression constructs in transient transfections were kept constant (2 μg). Error bars represent S. D. values calculated from three or more experiments. A control reporter (3xGAL4)-CR-CAT (with three GAL-4 binding sites substituting 21-base pair repeats in the human T - cell lymphotropic virus type I long terminal repeat) (34) trans-activated by GAL4VP16 (35) was not affected by ICER (10) (data not shown).

Abbreviations

IFN-γ, interferon-γ;

MIP-1α, macrophage inflammatory protein-1α;

MIP-1β, macrophage inflammatory protein-1β;

8-Br-cAMP: 8-Br-adenosine X, 5' - cyclic monophosphate

AICD, activation induced cell death;

AP-1: Activating protein 1

bZIP, basic region/leucine zipper;

BZTP: Basic region/leucine Zipper

cAMP, 3', 5' - cyclic adenosine monophosphate

CBP, CREB-binding protein

CD28RE, CD28 responsive element

CRE: cAMP response element

CREB: CRE binding protein

HAT, histone-acetyl-transferase

CREM: CRE modulator protein

DNA binding domain of NFAT, PBL, peripheral blood lymphocytes

EMSA: Electromobility shift assay

FasL, Fas ligand, GM-CSF, granulocyte-monocyte colony stimulating factor

GM-CSF: Granulocyte-monocyte colony stimulating factor

HTLV-I: Human T cell leukemia virus

ICER: Inducible cAMP early repressor

IFNγ: Interferon-γ

IL-12, interleukin-12

IL-13, interleukin-13

IL-2: Interleukin-2

IL-4: Interleukin-4

NFAT DBD: DNA binding domain of NFAT

NFAT: Nuclear factor of activated T cells

NFkB, nuclear factor kB

PBL, peripheral blood lymphocytes

PGE: Prostaglandin E

PGE_2, prostaglandin E_2

PKA: Protein kinase A

TcR, T cell receptor

PMA: 12-O-tetradecanoylphorbol 13-acetate

T helper 1, Th1

T helper 2, Th2

Detailed Description of the Preferred Embodiment

I. Therapeutic Blockade of ICER Synthesis To Prevent ICER-Mediated Inhibition of Immune Cell Activity

Downregulation of the immune response plays a major role in the progression of a number of human afflictions. For example, cancer cells or infectious agents may evade destruction by the host's immune cells by inhibiting or downregulating the activity of such cells. In particular, the immune response may be evaded by preventing the expression of genes encoding cytokines which are essential for mounting an immune response. The

present invention relates to the therapeutic use of antisense nucleic acids to downregulate the activity of the Inducible cAMP Early Repressor (ICER) protein, a protein involved in repressing immune cell activity.

A family of factors have been identified which modulate gene transcription through sites on promoters called cAMP response elements (CREs). They are therefore called CRE-binding proteins (CRE-BP). Most CRE-BP are constitutively expressed by cells, and possess a P-box domain which permits switches in their function by phosphorylation.

One subgroup of these factors, typified by CREB proteins but also individual CREM proteins such as CREM-t, possess glutamine-rich "Q domains" flanking the P box which mediate nuclear transcriptional activation after the P box is phosphorylated. Such P box phosphorylation is accomplished by several enzymes, including the cAMP-activated enzyme Protein Kinase A (PKA). Therefore, this "Q domain"-containing subgroup of CRE-BP includes constituitively expressed nuclear transcription activating factors which can be switched on by cAMP-induced phosphorylation.

In contrast, another subgroup of CRE-BP, typified by constituitively expressed CREM proteins such as CREM- , CREM- and CREM- , lack the activating gluamine-rich "Q" domains, and therefore function as nuclear transcription repressor factors; phosphorylation of their P-box via cAMP/PKA diminishes their activity.

While cAMP elevation can lead to phosphorylation but not de novo synthesis of the above two subgroups of CRE-BP, a third subgroup, the family of ICER isoforms, was discovered in contrast to be inducible by cellular cAMP elevation. Because all ICER isoforms lack a P-box, phosphorylation is unlikely to play as prominent a role in its function as in the P-box containing CRE-BP. In addition, because all ICER isoforms lack "Q" domains, they function as nuclear repressors. (Lalli, E. and P. Sassone-Corsi, 1994. "Signal transduction and gene regulation: the nuclear response to cAMP" J of Biol Chem 269: 17359-17362, the disclosure of which is incorporated herein by reference).

ICER mRNA is transcribed from an internal promoter located in an intron within the CREM gene. The ICER promoter contains four CREs in tandem and is highly inducible by cAMP in a variety of neuroendocrine cell lines, including AtT20 corticotrophs, GH3 and GC somatotrophs/lactotrophs, -TSH thyrotrophs, -T3 gonadotrophs, PC12 pheochromocytoma cells, and JEG-3 choriocarcinoma cells.

There are four isoforms of the ICER transcript, designated as ICER I, ICER I , ICER II, and ICER II , each of which encode proteins having a DNA binding domain. (Molina, C.A. et al., Cell 75:875-886, 1993, the disclosure of which is incorporated herein by reference.). These isoforms result from alternative splicing of the ICER transcript. The sequence of the full length ICER transcript which is differentially spliced to generate the four isoforms is disclosed in Biochemistry: 115:298-303 (1994), the disclosure of which is incorporated herein by reference, although at the time the sequence had not been given the name "ICER". Figure 1, which is derived from Figure 2 in Biochemistry: 115:298-303 (1994), the disclosure of which is incorporated by reference, lists the sequence of the full length ICER mRNA transcript with various domains of the mRNA indicated above the sequence. Figure 2, taken from Figure 6 of Gellersen et al., Molecular Endocrinology 11: 97-113 (1997), the disclosure of which is incorporated herein by reference, indicates the structures of the four ICER isoforms.

As illustrated in Figures 1 and 2, each isoform of translated ICER protein includes a bZIP DNA binding domain (DBD I or DBD II), ICER I/ICER I , isoforms containing DBD I and ICER II/ICER II , isoforms containing DBD II. The bZIP domain is present in a number of proteins which regulate transcription. Proteins containing bZIP domains are capable of forming heterodimers with other bZIP proteins which have unique DNA binding and transcriptional characteristics. By virtue of its bZIP domain and absence of "Q" domains, ICER is believed to heterodimerize with and block the activating properties of other nuclear transcriptional activators such as CREB. As described in more detail below, ICER can form ternary inhibitory complexes with the nuclear factor of activated T cell (NF-AT).

The ICER protein binds to CREs in the ICER promoter, thereby enabling autorepression of the ICER gene. In addition to its role as an autorepressor, ICER has also been shown to influence a variety of corticotroph functions. Unregulated ectopic expression of ICER produced drastic effects on corticotroph morphology. Forskolin treated cells expressing high levels of ICER are blocked in the G2/M phase of the cell cycle through downregulation of the cyclin A promoter mediated by ICER binding to CREs therein. (Lamas, M. et al., Molecular Endocrinology 11: 1425-1434, 1997, the disclosure of which is incorporated herein by reference). In contrast, expression of ICER antisense in these cells overcomes the G2/M block.

The present invention is based on the observation that, in addition to its activity in neuroendocrine cells such as corticotrophs, ICER may be involved in downregulating the activity of a wide variety of genes involved in stimulating the immune response. In particular, ICER binds to a number of recognition sites present in the promoters of genes encoding cytokines critical to the immune response. Accordingly, procedures for reducing the level of ICER expression in immune cells are useful both as therapeutic treatments to prevent downregulation of immune cell activity and for studying the mechanisms of regulation of immune cell activity. In particular, reduction in ICER expression levels may be used to modulate immune cell activity by bloaking repression of cytokine expression and/or other functions of cells of the immune system.

Initial experiments demonstrated that ICER expression was upregulated in medullary thymocytes or T cell lines treated with either forskolin or PGE2, both of which are known agonists of the cAMP signalling pathway. (Bodor, J. et al., Proc. Natl. Acad. Sci. USA 93: 3536-3541, 1996, the disclosure of which is incorporated herein by reference). ICER mRNA levels were elevated in T cells treated with both forskolin and the protein synthesis inhibitor cycloheximide relative to the mRNA levels observed in cells receiving forskolin alone, indicating that ICER may autorepress its promoter in T cells as well as in neuroendocrine cells.

The following examples further describe the regulation of ICER expression and the types of immune cells in which ICER is expressed.

Example 1

ICER Expression in T Cells can be Induced by Agents other than cAMP

Calcium mobilization, either through stimulation of the T cell receptor (TcR) or the addition of calcium ionophore (CI), can stimulate cytokine release and initiate events leading to proliferation in T lymphocytes. To maximize T cell activation and avoid anergy, additional costimulation is required, either in the form of pharmacologic

agents (Protein Kinase C activators like PMA/TPA), crosslinking of the T cell's CD28 molecule with anti-CD28 antibody, or the addition of antigen-presenting cells which express appropriate costimulatory molecules (2,3).

T cell proliferation and cytokine secretion can be inhibited by a variety of agents, including cAMP agonists, IL-10 and glucocorticoids (2,3). In the present example ICER mRNA induction in the presence of CI or the cAMP agonist forskolin was investigated. ICER mRNA induction in the presence of CI or the cAMP agonist forskolin was examined as follows.

Lymphocytes were obtained by leukapheresis/elutriation from normal human donors (4), and $CD4^+$ T cells were isolated using commercially-available negative immunoselection columns (4). These $CD4^+$ T cells were cocultured every two weeks with autologous monocytes which were pulsed for 24 hours with tetanus protein (TET) prior to coculture (4). rIL-2 (300 IU/ml) was added every 3-4 days to the coculture to promote outgrowth of TET-specific $CD4^+$ T cells. At the end of a growth cycle, the $CD4^+$ T cells were washed and rested in IL-2-free medium overnight. The next day, they were cultured in the following groups: No treatment; forskolin (100 micromolar); the CI ionomycin (1 microgram/ml), ionomycin plus forskolin. Normal anti-TETANUS human $CD4^+$ T cells were cultured for either 4 or 30 hours with no additive, forskolin (100 micromolar), ionomycin (1 microgm), or forskolin plus ionomycin. At the end of a four hour culture period cells were harvested and an RNAase protection assay performed as described in Bodor et al (5).

The results are shown in Figure 3. Predicted bands for ICER isoforms are identified at the right. Additionally occurring bands are believed to be artifacts resulting from polymorphisms where the samples' ICER sequence differs from the assay's ICER template (derived from the T cell leukemia Jurkat (5)).

As illustrated in Figure 3, either forskolin or ionomycin markedly induced ICER mRNA expression in $CD4^+$ T cells within 4 hours (Figure 3, lanes 1-4). After 4 hours of culture, ICER induction is prominent in ionomycin, forskolin and ionomycin plus forskolin treated groups. ICER I induction appears prominent for both forskolin and ionomycin, whereas other isoforms are prominently induced only in the presence of ionomycin. After 30 hours of culture, ICER induction is prominently sustained only in the group treated with both forskolin and ionomycin.

ICER expression in monocytes was investigated as described in Example 2 below.

Example 2

ICER Expression in Monocytes

Calcium ionophore (CI) induces dendritic cell (DC)-like differentiation of monocytes, including *de novo* expression of the costimulatory molecule B7.1 (4), U.S. Patent No. 5,643,786 (issued July 1, 1997), and U.S. Patent Application Serial No. 08/885,671 filed June 30, 1997, the disclosures of which are incorporated herein by reference. This is enhanced by the copresence of certain cytokines, such as GM-CSF (6). However, certain other agents inhibit CI-mediated differentiation of monocytes and DC, including cAMP agonists such as PGE2 and forskolin, interleukin-10, and glucocorticoids (6-11). Each of these inhibitory agents suppresses the induction of B7.1 expression seen in

monocytes with CI treatment (6). In the present experiment, the ability of each of these agents--either "inhibitory" or "stimulatory"-- to induce ICER mRNA in monocytes was studied.

Monocytes were obtained by leukapheresis/elutriation from normal human donors (4). They were freshly cultured in several groups: No treatment, forskolin (100 micromolar), prednisolone (10 micromolar), rIL-10 (1000 units/ml), GM-CSF (50 ng/ml), calcium ionophore (ionomycin 1 microgm/ml). After four hours of culture, the groups were harvested and an RNAase protection assay for ICER mRNA was performed as described in (5).

Fresh human monocytes were cultured for 4 hours with no treatment, forskolin (100 micromolar), prednisolone (10 micromolar), rIL-10 (1000 units/ml), rhGM-CSF (50 ng/ml), calcium ionophore (ionomycin 1 microgm/ml). After four hours of culture, the groups were harvested and an RNAase protection assay for ICER mRNA was performed as described in (5) Predicted bands for ICER isoforms are identified at the right. Additionally occurring bands are believed to be artifacts resulting from polymorphisms where the samples' ICER sequence differs from the assay's ICER template (derived from the T cell leukemia Jurkat (5)).

The results are illustrated in Figure 4. As illustrated in Figure 4, after 4 hours of culture, ICER induction is apparent in all treated groups (calcium ionophore > > IL-10 > forskolin > prednisolone > GM-CSF). ICER I induction is the most apparent induced isoform, although all isoforms are prominently induced with calcium ionophore.

The duration of ICER expression was studied as described in Example 3 below.

Example 3

Sustained ICER Expression

cAMP agonists (forskolin, PGE2, dibutryl cAMP) have been used by others to induce ICER expression in a variety of cells. In such experiments, ICER mRNA expression was transient, generally abating after several hours. The major mechanism responsible for this abatement appears to be that synthesized ICER protein binds to and inhibits its own promoter, thereby turning off ICER mRNA transcription (12). ICER protein itself appears to have a half life of several hours and undergoes rapid proteosomal degradation after undergoing ubiquination (13). Because of these observed metabolic tendencies, it would not be predicted that sustained ICER mRNA would be observed, since the presence of ICER protein would be expected to downregulate mRNA synthesis by repressing the ICER promoter.

Surprisingly, the data presented below indicate that in certain circumstances sustained ICER mRNA expression does occur, and that cells in these circumstances experience a functionally inhibited state. Sustained ICER expression was first evaluated in T Lymphocytes.

Anti-TET CD4$^+$ T cells were freshly prepared and expanded in culture as described above. After resting in IL-2-free medium overnight, the cells cultured with nothing, forskolin (100 micromolar), ionomycin (1 microgm/ml), or ionomycin plus forskolin were collected for ICER mRNA determinations (see above) at 4 hours and at 30 hours of culture.

In addition, microproliferation assays were performed as follows. After resting in IL-2-free medium overnight, the anti-TETANUS human CD4$^+$ T cells were stimulated with nothing, ionomycin (1microgram/ml), 50 u/ml

rhIL-2, TETANUS-pulsed autologous monocytes, or immobilized anti-CD3 (100 ng/well). Each treatment condition was tested with or without forskolin (100 micromolar). Tritiated thymidine (^{3}H-TdR) was added at 24 hours and the cells harvested at 40 hours to determine proliferation The cells were counted by a scintillation counter to determination cell proliferation during that period.

Although CD4$^+$ T cells treated with forskolin, ionomycin or ionomycin plus forskolin each expressed high levels of ICER at four hours of culture, only the ionomycin plus forskolin group continued to express high levels of ICER mRNA at 30 hours of culture (Figure 3 above).

The results of the microproliferation assay are shown in Figure 5. As illustrated in Figure 5, during the 24-40 hour period in culture, the ionomycin alone group displayed significant proliferation, probably facilitated by self-costimulation from B7.1 and B7.2 expression on the T lymphocytes themselves (not shown). In contrast, in highly reproducible experiments, the forskolin plus ionomycin group showed 70-90% inhibition of proliferation compared to the ionomycin group alone (Figure 5). Such inhibition could be partially reversed in a dose-dependent fashion when the PKA antagonist RPcAMPS was included in addition to forskolin and ionomycin (data not shown), supporting that the observed forskolin-induced inhibition was, as expected, at least partly PKA-dependent.

The above results demonstrate that the cAMP agonist forskolin potently inhibited proliferation induced by each of the tested stimuli.

Sustained ICER expresssion was also evaluated in monocytes as described in Example 4 below.

Example 4

Sustained ICER Expression in Monocytes

Fresh (never cultured) human monocytes were cultured for either 4 or 30 hours with no additive, forskolin (100 micromolar), calcium ionophore (ionomycin 1 microgm/ml), or forskolin plus ionophore either in 24 well plates for subsequent FACS analysis or in 6 well plates for ICER mRNA determinations.. At the end of the culture period cells were harvested and an RNAase protection assay was performed as described in Bodor et al (5). Cells were collected for ICER mRNA determinations (see above) at 4 hours and at 30 hours of culture. At 30 hours cells were also harvested from the 24 well plates for determination of their expression of the costimulatory molecule B7.1.

The results are illustrated in Figure 6. Predicted bands for ICER isoforms are identified at the right. Additionally occurring bands are believed to be artifacts resulting from polymorphisms where the samples' ICER sequence differs from the assay's ICER template (derived from the T cell leukemia Jurkat (5)).

As demonstrated previously (4), CI treatment alone induces DC-like differentiation of monocytes, including *de novo* B7.1 expression. Forskolin treatment, while having no detectable effect on monocyte B7.1 expression when added alone, markedly inhibited the upregulation of B7.1 expression by CI (not shown). After 4 hours of culture, ICER induction is prominent in ionophore, forskolin and ionophore plus forskolin treated groups. ICER 1 induction appears prominent for both forskolin and ionophore, whereas other isoforms are prominently induced only in the presence of ionophore.

After 30 hours of culture, ICER induction is prominently sustained only in the group treated with both forskolin and ionophore. ICER I and ICER II appear to be the two most prominent isoforms at 30 hours (Figure 6).

The above results demonstrate that, although transient transcription of the ICER gene can be demonstrated in T lymphocytes and monocytes by a variety of agents which result in either functional inhibition or stimulation (see above), it appears that such transient expression of ICER does not correlate to prolonged functional inhibition. Instead, where prolonged functional inhibition was observed, sustained high levels of ICER mRNA expression were also observed. Although it is conceivable that such sustained ICER mRNA expression may be occurring in the absence of high levels of ICER protein, it is likely that in this case high levels of ICER mRNA expression are accompanied by high levels of ICER protein translation.

Without being limited to a particular mechanism of action, it appears that such sustained ICER expression requires simultaneous stimulation through more than one signalling pathway: for example, both an inhibitory (cAMP agonist) and a stimulatory (calcium ionophore) signal may be necessay. Thus, as described below, therapeutic strategies which reduce or prevent sustained expression of ICER may be particularly efficacious.

The observation that sustained expression of ICER resulted when both cAMP-dependent and calcium-dependent pathways were activated suggests that, in contrast to previous studies of purely cAMP-dependent ICER induction (12), newly synthesized ICER protein can in select circumstances fail to negatively regulate the ICER mRNA promoter.

Others have also recently demonstrated the occurrence of sustained ICER expression in human endometrial stromal cells treated for 12 days with medroxyprogesterone acetate and relaxin (12x). However, in their studies, ICER appeared to lack its previously documented repressive effect on the decidual (dPRL) promoter, suggesting that even though ICER expression was sustained it was not functioning as a repressive agent in those cells (12x). The authors suggested that simultaneous phosphorylation of competitive activating proteins such as CREM- 2 and CREB may have blocked the repressive effects of ICER in their cells (12x). Without being limited to a particular mechaninsm of action, it is believed that such a competitive mechanism is not the explanation in the present studies, because sustained ICER expression in lymphocytes and monocytes correlates well to functional suppression of the cells. Instead, it appears that the dual stimuli of a PKA agonist (forskolin) and a calcineurin activator (ionophore) not only induced ICER transcription and translation, but also induced a selective blockade of ICER negative self-regulation which did not impact ICER's repressive effects on other gene promoters. If such a selective blockade in negative ICER self-regulation is due to induction of a previously unidentified nuclear regulatory protein(s), blocking translation of such a postulated regulatory protein could prove to be a potent therapeutic strategy, since it might render it possible to block sustained expression of ICER without interfering with cellular events which require transient ICER expression for homeostasis. Such a regulatory protein would function by interfering with negative ICER self-regulation without blocking ICER's ability to bind and repress other enhancer/promoters. For reasons of clarity, we will henceforth refer to this as the Repressor(s) of ICER Negative Self-regulation (RINSR).

As described in Example 5 below, in addition to being inducible in T lymphocytes and monocytes, ICER is also induced in NK cells.

Example 5

ICER Induction in NK Cells

Inducing cAMP in cultured NK lymphocytes inhibits NK target-directed lysis (14). This suggests that ICER is inducible in NK cells and may inhibit various NK functions. ICER induction in NK cells was evaluated as follows.

Donors were leukapheresed and elutriated to observe a lymphocyte rich fraction (4). The lymphocytes were cultured with anti-CD56 coupled immunoparamagnetic beads, washed, then applied to a magetized column to positively select for NK cells, which were subsequently eluted off the magnet (4). This resulted in NK cell purification to 95-98%. These cells were tested for ICER mRNA and protein expression at various time points (time 0, 24 hrs), with or without forskolin treatment to induce cAMP.

Positively immunoselected NK cells initially expressed ICER; in other experiments, it was found that NK cells purified instead by negative immunoselection did not express ICER, indicating that positive immunoselection itself induced ICER. However, such expression was transient. Within 24 hours in culture ICER levels had returned to baseline, but ICER expression could then be reinduced with forskolin treatment (not shown).

The above results demonstrate that ICER expression is inducible in NK cells and may account for the putative inhibitory effect of cAMP agonists.

ICER expression is also inducible in B cells, as described in Example 6 below.

Example 6

ICER Induction in B Cells

It has been shown that ICER expression is inducible by forskolin in X-50-7 and AKATA cell lines, which are transformed B cell lines (5). This suggest that ICER expression is inducible in normal B cells, since raising cAMP in B lymphocytes by diverse stimuli has been demonstrated by others to have a variety of inhibitory effects (15,16). In particular, treating lymphocytes with both the cAMP agonist PGE2 and immune complexes results in prolonged B cell unresponsiveness, whereas either treatment alone does not (15,16);

Without being limited to a particular mechanism of action, it is believed that such inhibition seen in the context of dual stimuli may closely parallel that seen when sustained ICER elevation is observed in T lymphocytes and monocytes following combined treatment with calcium ionophore and forskolin.

ICER expression in B cells was investigated as described in Example 7 below.

Example 7

ICER Expression in B Cells

B lymphocytes were freshly prepared to $>95\%$ purity from fresh human peripheral blood lymphocytes using positive selection with anti-CD20 coupled immunoparamagnetic beads (see above). Insufficient numbers were

obtained for culture, but the freshly isolated B cells expressed ICER protein, similarly to NK cells freshly isolated by positive immunoselection (see above).

The above results indicate that ICER expression occurs in normal B cells as well as transformed B cell lines, and may account for the putative inhibitory effect of cAMP agonists in several circumstances.

To confirm that ICER sustained expression of ICER protein occurs under conditions which cause sustained expression of ICER mRNA, the procedure of Example 8 is performed.

Example 8

Demonstration that Sustained Expression of ICER Protein Occurs Under Conditions which cause Sustained Expression of ICER mRNA

Cells are exposed to combined stimuli which induce sustained expression of ICER mRNA and which cause strong functional inhibition, such as forskolin plus ionomycin. Protein samples are obtained from the cells and levels of ICER protein are determined using an antibody which recognizes the ICER protein. ICER protein levels in cells in which which sustained expression of ICER has been induced are compared to those of uninduced cells. The results indicate that ICER sustained expression of ICER protein occurs under conditions which induce sustained expression of ICER mRNA.

II. Role of ICER in Cyclic AMP-Mediated Attenuation of Cytokine Gene Expression in Human Thymocytes

It is well established that cAMP signaling is inhibitory to T cell proliferation and effector functions. In particular, cAMP inhibits the expression of T helper-1 cytokine genes (30-32). Earlier reported studies of fibroblasts showed that elevated levels of intracellular cAMP inhibit upstream signal transduction pathways involved in cell growth and differentiation (33, 34). In contrast to fibroblasts, in which elevated levels of intracellular cAMP inhibit ERK1, ERK2, and JNK kinases involved in the signal transduction of MAP kinase pathways, T cell ERK1 and ERK2 are insensitive to elevated levels of intracellular cAMP (35). Moreover, the cAMP-mediated inhibition of JNK kinase in T cells shows delayed kinetics, an observation that correlates with the induction of the cAMP-inducible early repressor ICER (36). In addition, overexpression of NFAT achieved by transfection of NFAT-encoding cDNAs to lymphoma cells abrogates the sensitivity of cAMP-mediated inhibition of IL-2 gene expression (37,38). Importantly, phosphorylation of amino-terminal serines of NFAT by PKA does not prevent calcineurin-mediated translocation of NFAT to the nucleus, despite its ability to prevent IL- 2 gene expression (39,40). The notion that a newly synthesized transcriptional repressor rather than inhibition of upstream signal transduction pathways could be involved in the cAMP-mediated transcriptional attenuation of T helper-1 cytokine expression was further strengthened by the reported alleviation of cAMP-mediated inhibition of IL-2 expression in the presence of inhibitors of both RNA and protein synthesis (35).

As discussed above, ICER is a transcriptional repressor that appears to serve as a generalized negative regulator of the CREB and CREM family of transcription factors as well as other related bZIP family members (41-44). ICER isoforms represent a unique cAMP-inducible CREM subfamily of transcription factors containing cAMP response elements within an internal P2 promoter. Because of autoregulation of the cAMP-inducible P2 promoter, the expression of ICER can be intrinsically rhythmical. The rhythmical expression of ICER was first described in the pineal

gland and in the hypothalamic-pituitary-gonadal axis (45,46). However, the P2 promoter of ICER is also inducible in organs other than the pineal and hypothalamic-pituitary gonadal axis such as in specific subsets of T lymphocytes including human medullary thymocytes (36). Importantly, in the Jurkat T cell line ectopically expressed ICER can substitute for the inhibitory effects of cAMP on the transcriptional attenuation of IL-2 promoter activity (36).

The Nuclear Factor that Activates T cells (NFAT) and Activating Protein 1 (AP-1) represent two major transcription factor families implicated in the transcription of the IL-2 promoter in proliferating T lymphocytes (47-49). To address the possible mechanism by which ICER downregulates IL-2 gene expression the binding of bacterially expressed ICER to all five NFAT motifs of the IL-2 promoter reported to be essential for the full induction of the IL-2 gene [50] either alone or in the presence of the minimal DNA-binding domain of NFAT (NFAT DBD) was examined. The highest affinity of ICER binding was found on a CD28-responsive element (CD28RE; -160 NFAT/AP-1 composite site) and (-90) site which is the motif in the IL-2 promoter that has striking sequence homology with the conserved proximal region (-73 to -48 bp) of both the human and mouse promoters of the IFNγ genes (50,51).

Moreover, certain NFAT/AP-1 composite sites that reside within the IL-4, GM-CSF, and TNF-α promoters resemble those located within the IL-2 promoter (52-56). It is believed that the mechanism underlying the actions of NFAT requires the binding of NFAT and/or NFAT/AP-1 to the NFAT/AP-1 composite binding motifs as ternary complexes (47). These complexes are believed to be essential for the transcriptional expression of immunoregulatory cytokines during T cell proliferation, such as IL-2, IL-4, GM-CSF, and TNF-α (48).

As shown below, ICER binds to these NFAT/AP-1 composite DNA sites *in vitro*, either directly or indirectly via complex formation with the rel homology region of NFAT (NFAT DBD). Furthermore, induction of ICER-immunore active complexes was detected in extracts prepared from human medullary thymocytes treated with forskolin and ionomycin. Ectopically expressed ICER represses transcription from the IL-2, GM-CSF, and TNF-α promoters activated by ionomycin and phorbol ester, suggesting that the induction of ICER in response to cAMP may be responsible for the observed cAMP-mediated transcriptional attenuation of T helper-1 cytokine responses.

The following techniques and procedures were used in the experiments described below.

Preparation of human medullary thymocytes

Human thymus glands were obtained from children (ages 3 months to 4 years) undergoing corrective carliac surgery. Thymocytes were fractionated over discontinuous Percoll gradients (Pharmacia) (57). Cells with densities of $1.060 < o < 1.070$ and $P > 1.070$ were collected and classified into large (significantly enriched for medullary thymocytes) and small (cortical) thymocytes according to an established protocol (36). Separated human thymocytes were maintained in short-term cultures in RPMI 1640 medium, supplemented with 10% fetal calf serum, 50 units/ ml penicillin, and 25 μg/ml streptomycin, and treated as indicated.

RNase Protection Analysis

RNA extraction was performed as described (Qiagen). RNA probes hCK1 and hCK3 were purchased from Pharmingen and labeled with [α-^{32}P]CTP using reagents from an RNA probe kit (Ambion). These probes were used for RNase protection studies according to the protocol provided by Ambion (RPAII Ribonuclease Protection Assay kit).

Western blot analysis

Separation of whole cell proteins (50 μg) was carried out by SDS-polyacrylamide gel electrophoresis (10%) for 2 hours at 40 mA in Tris-glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) at room temperature. The proteins were electrotransfered (0.4 A) overnight at 4°C in 10 mM Tris glycine buffer with 12% methanol on Immobilon P membrane (Millipore). The membrane was blocked in a Tris-buffered saline solution containing 0.05% Tween 20 (Sigma) and 5% nonfat dry milk (Bio-Rad) for 1 h with gentle agitation at room temperature. For immunological detection, the same solution without dry milk but containing the ICER or CREM-specific antiserum (CS4) diluted 1:10,000 was agitated for 1 h, followed by three washes, with subsequent incubation with horseradish peroxidase conjugated to a secondary antibody (Amersham) diluted 1:5,000 for 1 h, followed by nine washes and finally developed using an ECL kit (Amersham).

Expression and purification of recombinant proteins

Human ICERII cDNA was subcloned into the pGEXKG vector (Pharmacia) and expressed in bacteria as a GST-fusion protein. The pGSTagCREB construct was described previously (58). Purifications of both ICER and CREB were carried out with minor modifications according to the protocol previously established for CREB (58). NFATpXS(1-187) encompassing the minimal DNA-biding domain of NFATp was expressed in bacteria as a hexahistidine-tagged protein and purified as reported previously (59). Recombinant c-Fos (Fos 139-243) and c-Jun (Jun 187-334) were purified from E coli overexpression strains by nickel chelate affinity chromatography (60, 61).

Nuclear extracts and gel mobility shift assay

Whole cell extracts were prepared by high salt extraction using 50 mM Hepes, 250 mM NaCl, 5 mM EDTA, 0.5 mM DTT and protease inhibitors, (20 μM leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride) as described previously (59). Binding reactions were performed in a 15 μl reaction volume containing 20 mM HEPES, 1mM MgCl2,50 mM KCl, 12% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.2 μg poly (dI-dC) as an unspecific competitor, and recombinant proteins or whole cells extracts as indicated. 32P-labeled oligonucleotides and, where indicated, unlabeled competitor oligonucleotides in excess were added and incubated for 10 min at room temperature. Samples were run on a 4% polyacrylamide gel in 0.5 x TBE at 200 V for 2 h following 2 h pre-run at 4 degrees C. The dried gels were exposed for autoradiography overnight. The oligonucleotides encompassing NFAT composite sites of the following human promoters were used:

SEQ ID NO. 2: IL-2 (-45) 5'-ctagaCATTTTGACACCCCCATAATATTTTTCCAGAATTa-3';

SEQ ID NO. 3: IL-2 (-90) 5'-ctagaGTCTTTGAAAATATGTGTAATATGTAAAACATa-3';

SEQ ID NO. 4: IL-2 (-135) 5'-ctagaATCAGAAGAGGAAAAATGAAGGTAATGTTTTa-3';

SEQ ID NO.5: IL-2 (-160) 5'-ctagaAAAGAATTCCAAAGAGTCATCAGAAa-3';

SEQ ID NO. 6: IL-2 (-280) 5'-ctagaAAGAAAGGAGGAAAAACTGTTTCATACAGa-3';

SEQ ID NO. 7: GM-CSF(-330) 5'-gatccCCCCATCGGAGCCCCTGAGTCAGCATGGa-3';

SEQ ID NO. 8: GM-CSF (-420) 5-gatccCATCTTTCTCATGGAAAGATGACATCAGGGAa-3';

SEQ ID NO. 9: GM-CSF (-550) 5'-gatccGAAAGGAGGAAAGCAAGAGTCATAATAAGAa-3';

SEQ ID NO. 10: IL-4 (-80) 5'-gatccTAACTGCAATCTGGTGTAATAAAATTTTCCA IGTAAA

SEQ ID NO. 11: CTCATa-3'; TNF-α (-95) 5'-gatccTTCCTCCAGATGAGCTCATGGGTTTCTCCACGACGGAa-3'.

Lower case letters indicate overhangs for SpeI/XbaI recognition sites (IL-2) or BamHI/BglII recognition sites (GM-CSF, IL-4, TNF-α). As competitors the following oligonucleotides were used: nf (mouse IL4 NFAT, positions -69 to -79) SEQ ID NO. 12: 5'-ATAAAATTTTCCAATGTAAA-3'; ap (human metallothionein IIA AP-1 site [MREI, positions -114 to -88) SEQ ID NO. 13: 5'-GAGCCGCAAGTG ACTCAGCGCGGGGCG-3', and cre (mouse c-fos gene oligonucleotide, surrounding CRE site in position -60) SEQ ID NO. 14: 5'-gatccCAGTTCCGCCCAGTGACGTAGGAAGTCCATCa-3'. Lower case letters indicate overhangs for BamHI / Bgl 11 recognition sites.

GST pull-down assays

GST-ICER (GST-CREB) sepharose beads prepared as described above were diluted 1:10 in 50 mM Tris pH7.5,150 mM NaCl, 0.5 mM EDTA, 10 mM Na3 (PO4)2, 10 mM NaF, 0.1% Triton X100, 2.5 mM Leupeptin, 20 mM PMSF, 100 μg/ml Aprotinin, recombinant protein(s) added to a final volume of 250 μl, and incubated at 4 degrees C on a nutator for 1.5 hrs. The beads were then washed 3x with the same buffer, resuspended directly in Laemli buffer, and loaded on 10% SDS-PAGE gel. Retained NFAT DBD protein was visualized by Western blotting described below using R59 anti-NFAT DBD antiserum.

Transient overexpression in Jurkat T cells

Transfection assays were performed by the DEAE-dextran technique. Typically, 10^7 Jurkat cells were transfected with 2 μg of the reporter, the same amount of ICER expression vector and treated 18 hrs posttransfection with phorbol ester (10^{-5} mg/ml) and ionomycin (1 μg/ml) for 48 hrs. Luciferase and chloramphenicol acetyltransferase assays and quantification methods are described elsewhere (Promega, (62)). The percent conversion of [^{14}C]chloramphenicol to its acetylated forms was quantified using ImageQuant (Molecular Dynamics).

Example 9

cAMP-mediated Transcriptional Attenuation of T helper-1 Responsive Cytokine Genes in Human Medullary Thymocytes

Under the experimental conditions used human medullary thymocytes exhibited the characteristics of naive T helper-0 (Th0) and T helper-1 (Th1) cells with predominant IL-2 and IFNγ ression documented by MIRNase protection used for the evaluation of mRNA levels of multiple cytokines (IL-2, IL-4, IL-5, IL-9, IL-10, M-13, IL-14, IL-15, IFNβ, IFNγ, MIF, TNF-α, Ltβ, TGFβ1, TGFβ2, and TGFβ3) (Fig. 7). Stimulation of medullary thymocytes with a combination of phorbol ester and ionomycin significantly induced the synthesis of mRNAs encoding IL-2, IFNγ and to a lesser extent also TNF-α, and Ltβ (Fig. 7A; lane 4, Fig. 7B; lane 4). At the same time, cotreatment with forskolin or 8-Br-cAMP reduced the cellular mRNA levels of IL-2, IFNγ, TNF-α, and Ltβ (Fig. 7A; lane 5 and 6, Fig. 7B; lane 5 and 6). It is believed that at least part of this transcriptional attenuation is based on cAMP-mediated expression of the transcriptional repressor ICER and a subsequent blockade of NFAT/AP-1 composite DNA sites essential for T helper-1 cytokine expression. The inhibition by ICER may occur either directly through binding to the DNA element or indirectly via protein-protein interactions such as to the rel homology domain of NFAT (NFAT DBD).

Example 10

Cyclic AMP-mediated Attenuation of T helper-1 Cytokine Transcription in Human Medullary Thymocytes Correlates with cAMP-mediated Induction of the Transcriptional Repressor ICER

To test the mechanism involved in ICER-mediated inhibition, the presence of ICER protein in human medullary thymocytes after forskolin treatment was investigated to explore whether ICER interacts with important NFAT/AP-1 enhancer motifs of the IL-2, and IFNγ promoters. Western immunoblotting analysis using an ICER-specific antiserum (36) confirmed that ICER protein is indeed detectable in human medullary thymocytes but is absent in cortical thymocytes after 3 hrs of forskolin treatment (Fig. 7C). This finding is in agreement with the previously observed delayed appearance of ICER mRNA in medullary thymocytes after their exposure to forskolin and its subsequent de-induction (36). These observations indicate that the cAMP-mediated inhibition of cytokine expression may occur in a stage-specific manner in cells of T cell lineage.

Example 11

ICER Binds to NFAT/AP-1 Composite Sites of the IL-2 promoter:

To further address the mechanism by which ICER downregulates IL-2 gene expression the binding of bacterially expressed ICER to all five NFAT motifs of the IL-2 promoter reported to be essential for the full induction of the IL-2 gene [50] was examined (Fig 8A). Four out of five NFAT sites in positions (-90), (-135), (-160), and (-280) were previously characterized as NFAT/AP-1 composite sites due to their inherent ability to bind to the NFAT/AP-1 complex in a cooperative fashion (63). The fifth NFAT site in the most proximal position NFAT-45 does not bind to the NFAT/AP-1 complex and was determined to be exclusively an NFAT binding site (63). Bacterially expressed ICER, as well as ICER expressed in Cos cells (data not shown), binds to all five NFAT sites, although to different extents (Fig. 8B). The strongest binding was observed to the NFAT/AP-1 composite sites in positions (-90) and (-160), an intermediate binding to the (-135) NFAT/AP-1 composite site, and weak binding to the most proximal and distal NFAT and NFAT/AP-1 sites in positions (-45) and (-280), respectively. The binding specificity of recombinant ICER was evaluated using a CREM-specific antiserum (CS4) that "supershifts" ICER bound to specific oligonucleotides containing individually the five DNA motifs, leaving nonspecific binding unaffected (Fig. 8B, lanes 1-10) as well as by using control oligonucleotide encompassing the first 21 bp repeat (H21) of the HTLV-I LTR promoter containing the CRE-like motif (64) (Fig. 213, lanes 11 and 12).

Example 12

ICER Binding to NFAT/AP-1 Composite Sites in the Presence of NFAT DBD

To better understand the role of the interactions of ICER with NFAT/AP-1 composite DNA motifs, the effectiveness of ICER binding to the DNA motifs in the presence of the minimal DNA-binding domain of NFAT (NFAT DBD) was examined. This domain of NFAT, which has the highest degree of conservation amongst NFAT family members (65), is both necessary and sufficient for DNA-binding as well as to associate AP-1 with NFAT (59). All NFAT motifs on the IL-2 promoter examined, with the exception of the NFAT/AP-1 composite site in position (-90), bound NFAT efficiently

(Fig. 8C). The most pronounced ability of NFAT to associate with ICER was observed on the sites with the highest DNA-binding efficiencies to both proteins, particularly at site position (-160) (CD28RE) and to a lesser extent to the NFAT site in position (-45). Interestingly, this NFAT -45 site tends to show a stronger binding of ICER to NFAT in the NFAT ICER complex compared to ICER by itself (Fig. 8C, lanes 1-3). Because the NFAT -45 of the IL-2 promoter is the only one of the five sites examined without an adjacent AP-1 site, the finding that this site can form an NFAT/ICER complex suggests that ICER itself may tether to NFAT by a protein-protein interaction in addition to a protein-DNA action.

Example 13

ICER Can Interact Directly with NFAT DBD

To examine whether or not ICER and NFAT may directly interact in the absence of DNA, GST-pull down assays were performed using a GST-ICER fusion-protein linked to a Sepharose matrix in the presence of a truncated NFAT consisting of the DNA-binding domain (NFAT DBD) (Fig. 9, lane 1). GST-ICER formed a complex with NFAT DBD as demonstrated by retention of NFAT DBD. Interestingly, GST-CREB failed to associate with NFAT DBD under the same conditions (Fig. 9, lane 10). To examine the specificity of both components in the NFAT/ICER ternary complex found on the CD28RE (-160 motif) of the IL-2 promoter both CREM-specific antisera (CS4) and antisera raised against the minimal DNA-binding domain of NFAT (R59) were used. Both antisera either prevented or reduced the amount of the NFAT/ICER complex (Fig. 8D, lanes 4 and 5, respectively). Competition with unlabeled oligonucleotides containing NFAT (nf oligonucleotide spanning mouse IL-4 NFAT, positions -69 to -79) or the AP-1 motif (ap oligonucleotide spanning the human metallothionein IIA AP-1 site [MRE], positions -114 to -88) eliminated the NFAT/ICER complex (Fig. 8D, lanes 6 and 7, respectively), suggesting that both ICER and NFAT are essential components of the observed complex.

Example 14

NFAT/ICER Ternary Complex Shows Distinct Features in Comparison with NFAT/AP-1 Complex on Various NFAT Motifs of IL-2 promoter

Analogous experiments performed with the NFAT DBD and truncated forms of Fos and Jun, confirmed in vitro that both NFAT/AP-1 and NFAT/ICER complexes could exist in the context of the CD28RE (-160 NFAT/AP-1 composite site; Fig 8D, lanes 8-14). Interestingly, in the presence of NFAT DBD, the majority of ICER protein was retained in the complex with NFAT DBD, although, NFAT/ICER and NFAT/AP-1 complexes appear to show unequal binding affinities to different NFAT/AP-1 motifs. For example, the (-280) motif of the IL-2 promoter, known from NFAT studies as the principal site for binding of the NFAT/AP-1 complex, binds NFAT/AP-1 complexes effectively (61,66) showing little or no detectable formation of an NFAT/ICER complex (Fig. 8C, lanes 13-15). In contrast, the CD28RE (-160 NFAT/AP-1 composite site) of human IL-2 promoter creates a ternary NFAT/ICER complex with an equal or slightly higher efficiency than the NFAT/AP-1 complex (Fig. 8D).

Example 15

ICER Binds Directly to the Conserved Proximal Motif of IFN-γ Promoter:

Unlike the CD28RE (460 motif) which shows equally high affinity for both ICER and NFAT, the NFAT/ AP-1 motif of the IL-2 promoter in position (-90), which has striking homology to the conserved proximal element of the IFNγ promoter, does not interact with NFAT DBID (Fig. 10). Studies performed on the conserved proximal motifs of both human and mouse IFNγ promoters demonstrated high affinity ICER binding and a lack of NFAT binding or NFAT/ICER complex formation in the presence of NFAT/DBD (Fig. 10): a situation similar to that observed on the homologous (-90) motif of the IL-2 promoter (Fig 8).

Example 16

NFAT/AP-1 Composite Sites in the context of the GM-CSF, IL-4, and TNF-α Promoters Bind ICER Either Alone or in Complexes

NFAT/AP-1 binding sites have been shown previously to be essential for the efficient activation of the GM-CSF, IL-4, and TNF-α promoters. Therefore the binding to these sites of ICER and NFAT, both as purified recombinant proteins and in extracts prepared from human medullary thymocytes treated with forskolin and ionomycin was examined (Fig. 11). These studies demonstrated that ICER can bind either by itself or in complexes with NFAT DBD to these composite sites in the promoters of the GM-CSF, IL-4, and TNF-α promoters, similar to the experiments using the binding site motifs of the IL-2 and IFNγ promoters.

The GM-420 DNA motif strongly bound the purified NFAT/ICER complex (Fig. 11B, lane 6), whereas the GM-330 and GM-550 motifs bound the complex much more weakly (Fig. 11B, lanes 3 and 9). It is noteworthy that the GM-420 motif has been shown to constitute the essential enhancer core of the GM-CSF promoter (52). Likewise, NFAT/ICER readily formed a complex with the -80 element of the IL-4 and -95 element of the TNF-a promoter (Fig. 11B, lane 12 and 15, respectively). Interestingly, the κ3 motif of the TNF-α promoter, which contains an "'inverted CRE" motif adjacent to the NFAT composite site (27), created a complex with an electrophoretic mobility different from those observed on the motifs of the IL-2, IL-4, and GM-CSF promoters (Fig. 8C and Fig. 11B). Furthermore, NFAT DBD alone formed a much slower mobility complex (lane 14), suggesting that NFAT may bind to the TNF-α motif as oligomers.

The treatment of isolated human medullary thymocytes with forskolin and ionomycin readily induced the expression of ICER (Fig. 11D and 11E) not seen in uninduced thymocytes (Fig. 11C). The binding of ICER to the oligonucleotides containing the NFAT/AP-1 composite sites is inhibited by competition of the binding with a CRE-containing oligonucleotide (Fig. 11D, lanes 4, 7 and 10) or interference of the binding with antiserum to ICER (C-Ab) (Fig. 11E, lanes 4, 7, and 10).

Example 17

Ectopically Expressed ICER Represses NFAT-mediated Activation of IL-2, GM-CSF, and TNF-α promoters

To determine whether ICER expression could supplant the effect of forskolin in transcriptional attenuation of various cytokine promoters observed in medullary thymocytes, ICER (isoform II) was expressed in Jurkat T cells in transient transfection assays. Expression of ICER downregulated the human IL-2, CM-CSF, and TNF-α promoters

activated by the combined treatment of the cells with PMA and ionomycin, whereas ectopic expression of neither isoform of ICER did not prove to have any significant effect on VP16-mediated transactivation of (3xGAL4)-CR-CAT under the same conditions (Fig. 12). Thus, ICER can be induced by, and substituted for, forskolin in the transcriptional downregulation of the calcineurin-dependent, NFAT/ AP-1-mediated transactivation of IL-2, GM-CSF, and TNF-α promoters, when induced by PMA and ionomycin.

The mechanism of cAMP-mediated inhibition of cytokine expression in proliferating T lymphocytes has been attributed to cAMP-mediated inactivation of upstream signal transduction pathways directing the proliferation of T lymphocytes. Although this hypothesis was supported by studies of fibroblasts (34) it was not confirmed by studies of T cells (35). Surprisingly, several protein kinases required for T cell proliferation were found to be insensitive or to exhibit a delayed response to high levels of intracellular cAMP (35). The above results indicate that in human medullary thymocytes expression of the transcriptional repressor ICER correlates with a delayed cAMP-mediated transcriptional attenuation of T helper-1 cytokine responses.

Footprinting and electrophoretic mobility shift analysis of the IL-2 promoter revealed (63) that the originally defined AM site at position -150 of the IL-2 promoter (66,67) a major CD28 responsive element (CD28RE) that contains an upstream NFAT binding site (68, 69), represents a novel NFAT/AP-1 composite site at position -160 (63). A re-examination of the original observations in which NFκB was identified as a major component of the complex (70,71) determined that NFAT is a prevalent component of the complex that binds to CD28RE in vivo (63,72). These findings indicate that the CD28RE (-160 composite site of IL-2 promoter) effectively binds ICER either alone or in an NFAT/ICER complex. These findings may be important for obtaining a better understanding of a direct cAMP-mediated transcriptional attenuation of IL-2 expression. In addition, a potential indirect role of ICER has been demonstrated in transgenic mice overexpressing the dominant negative CREB mutant (a functional homologue of ICER), which impairs the expression of IL-2 in thymocytes (73). Since the induction of IL-2 and IFNγ expression is dependent on the activity conferred by each of the individual DNA motifs (51, 63), demonstration of a direct binding of ICER and/or the formation of an inhibitory NFAT/ICER complex on any of these NFAT/AP-1 composite sites could provide an explanation for the mechanism involved in the transcriptional attenuation of IL-2 and IFNγ expression mediated by cAMP. These findings correlate with observations in which the conserved proximal motif of the IFNγ promoter was reported to be inhibited by forskolin in proliferating thymocytes of mice made transgenic with an IFNγ promoter-luciferase reporter gene (74). These findings further suggest that both NFAT/AP-1 motifs, either those that directly bind ICER or those that form NFAT/ICER complexes, could convey ICER-mediated transcriptional attenuation.

It appears that numerous NFAT/AP-1 composite sites previously identified in the context of GM-CSF, IL-4, and TNF-α promoters as essential determinants of their expression (52, 54, 56) can associate with ICER. This property does not seem to be a universal feature shared by all NFAT/AP-1 composite sites tested because relatively minor differences in DNA sequences have a profound effect on both the binding of ICER and the formation of NFAT/ICER complexes. An example of this circumstance is represented by the GM-CSF promoter in which the GM-420 element shows binding for ICER and NFAT/ICER complexes, whereas both neighboring motifs GM-330 and GM-550

show only a modest binding of ICER and/or formation of NFAT/ICER complexes. The strong binding of ICER to the GM-420 element, previously defined by deletion analysis as the essential core of the GM-CSF enhancer (54), suggests that ICER may play an important role in transcriptional attenuation of GM-CSF expression. Similar binding studies performed with several NFAT/AP-1 composite sites important in the context of IL-4 and TNF-α promoters show that these sites, previously shown to be essential for efficient expression (52, 54, 56), bind ICER either alone or in complexes similarly to the motifs of the IL-2 and IFNγ promoters. It remains to be determined whether the induction of ICER can selectively modulate T helper-1 versus T helper-2 cytokine expression in peripheral lymphocytes.

It has been reported that human medullary but not cortical thymocytes synthesize ICER mRNA after three hours of forskolin treatment (36). Western immunoblot analysis using an ICER-specific antiserum confirmed that in these conditions the ICER mRNA is translated efficiently into ICER protein. Moreover, endogenously expressed ICER protein was detected in extracts prepared from human medullary thymocytes treated with forskolin and ionomycin using oligonucleotide probes containing NFAT/AP-1 DNA motifs that are able to form NFAT/ICER complexes in vitro. In contrast to bacterially expressed ICER, endogenously expressed ICER in medullary thymocytes shows an altered mobility in gel shift assays, suggesting that posttranslational modification(s) may be involved in the regulation of the binding properties of ICER and/ or degradative pathways involved in its proteolysis *in vivo*. The ICER-containing complexes that are immunoreactive to ICER-supershifting antisera are efficiently competed by oligonucleotides containing CRE or NFAT motifs. NFAT antisera which are unable to recognize directly bound ICER still affect the mobility of ICER-containing complexes, suggesting the possibility of the formation of NFAT/ICER complexes *in vivo*. The ambiguity of the DNA-protein complexes in extracts of thymocytes observed on gel shift assays may be due to posttranslational modifications of the proteins involved (data not shown) and/or their potential consequences for DNA-binding. At this point it cannot be excluded that proteins other than ICER and NFAT which contain homologous bZIP or rel homology regions may also participate in the formation of ICER-containing complexes. Finally, ectopic expression of ICER in jurkat cells demonstrates that ICER, in agreement with its binding capabilities, can also effectively inhibit NFAT-mediated, phorbol ester/ ionophore induced expression of IL-2, GM-CSF, and TNF-α promoters.

Thus, inducible ICER expression in developing human medullary thymocytes as well as in certain subset(s) of human peripheral blood lymphocytes (36) and monocytes (work in progress) could significantly influence their respective effector functions(s). The proposed inhibitory effects on effector function of the immune system mediated by ICER may be related to its ability to bind (mask) a wide range of CRE and AP-1 motifs and/ or its ability to inactivate certain transcription complexes via protein-protein interactions.

III. Suppression of T Cell Effector Functions via ICER-Mediated Transcriptional Attenuation of Cytokine and Chemokine Expression

ICER (inducible cAMP early repressor), the only cAMP-inducible member of the CREB/CREM family of transcription factors, is a potent regulator acting in response to the cAMP/PKA signal transduction pathway to repress expression of specific cytokine genes in T lymphocytes (76, 77). Depending on the nature of costimulation which is critical for the interleukin-2 (IL-2) production, expression of numerous regulatory cytokines such as IFN-γ, IL-

4, IL-5, IL-10, and IL-13 can be either susceptible or resistant to cAMP-mediated inhibition in human peripheral blood T lymphocytes polarized toward T helper 1 and T helper 2 dominant phenotypes *in vitro*. In the absence of IL-2, forskolin a well known cAMP-elevating agonist, causes a dramatic transcriptional attenuation of early expression of numerous immunoregulatory cytokines and chemokines. This cAMP-mediated transcriptional attenuation tightly correlates with induction of ICER. To prove that ICER *per se*, rather than forskolin-mediated elevation of intracellular cAMP, is responsible for the observed inhibitory effect, transgenic mice expressing ICER under the control of lymphocyte-specific *lck* promoter were generated. Upon stimulation, transgenic thymocytes expressing high levels of ICER failed to express IL-2 and IFN-γ as well as MIP-1α and MIP-1β. Moreover, T cells from transgenic spleen exhibited conditional defect in proliferation resembling anergy accompanied by the lack of allogeneic response in mixed lymphocyte reaction suggesting that ICER-mediated transcriptional attenuation of cytokine and chemokine expression may play an important role in inactivation of T cell effector functions.

ICER (inducible cAMP early repressor) belonging to the CREB (78) and CREM (79) family of the basic-leucine zipper transcription factors (80), acts as a dominant negative regulator of the cyclic AMP-dependent protein kinase A (cAMP/PKA)- signal transduction pathway (81). ICER was originally described in the pineal gland as a powerful repressor of cAMP-induced transcription driven by rhythmic adrenergic signals originated from the endogenous clock (82) and subsequently implicated in the regulation of several physiologic functions of the hypothalamo-pituitary-gonadal axis (for review see (83)). Originally, ICER has been thought to be expressed exlusively in the hypothalamo-pituitary-gonadal axis until discovered in the immune system where it was proposed to act as a potent modulator of T cell effector functions (76).

Importantly, ICER represents the only known cAMP-inducible subfamily among CREB/CREM transcription factors. ICER consists of four different isoforms generated by alternative splicing of its transcript (ICERI, ICERIγ, ICERII, ICERIIγ)(81), with alternating exon-γ, encoding either CREB-like (ICERI) or CREM-like (ICERII) DNA-binding domains. Alternative splicing allows, after translation, a promiscuous binding of ICER proteins to a wide variety of cyclic responsive elements (CREs) and CRE-like DNA motifs which are regularly occupied by ubiquitiously expressed CREB and/or related transcription factors containing homologous basic leucine zipper (bZIP) domains such as members of CREM, ATF, Fos, and Jun families (84, 85). However, ICER subfamily represents a natural truncation of full length CREM due to its use of an alternative P2 promoter located in the middle of CREM gene. Therefore, ICER subfamily lacks the upstream transactivation domain of CREM transcription factors, since transcription of ICER starts downstream of the transactivation domain of CREM and possesses mainly CREB-like or CREM-like DNA-binding domains (81). Lack of CREM transactivation domain, highly homologous to transactivation domain of CREB, is believed to be responsible for failure of ICER to recruit CREB-binding protein/p300 (CBP/p300) (86, 87) which predetermines ICER to serve as a potent transcriptional repressor (88) opposing CREB mediated transcription (80).

There are more than 1,200 different G protein coupled seven-span transmembrane receptors exhibiting specificity for diverse array of ligands, such as prostanoids, chemokines, and hormones, many of them, including prostaglandin E_2 (PGE_2) receptor, are present on the surface of T cells (89). These receptors often activate

cAMP/PKA as well as numerous other signal transduction pathways leading to phosphorylation on the Ser-133 residue of CREB (90-92) which allows phospho-CREB (or its homologues (93, 94)) anchored on CREs of different promoters to recruit CBP/p300 complex involved in the variety of T cell responses including proliferation, differentiation or hormonal responses (86, 95, 96). However, at the same time, ICER can be induced in parallel through CRE-like autoregulatory responsive elements (CAREs) located in its intronic P2 promoter in the middle of CREM gene (97). Once expressed, ICER protein has a capacity to compete with CREB for binding to numerous CRE-like DNA binding motifs including ICER's own CAREs in the P2 promoter, eventually leading to its own downregulation. There are many known promoters critically involved in proliferation containing CRE motifs, for example CRE motif of the c-fos promoter in the position (-60) (88, 98). Therefore, it is possible that ICER may first inhibit genes involved in T cell activation and/or proliferation (99) before shutting down its own expression (97). Hence, the stability, binding, and trafficking of ICER protein in combination with inherently cyclical nature of its expression might explain delicate balance directing conditional character of cAMP-mediated transcriptional attenuation.

In spite of many P1 promoter initiated spliced variants of CREM, ICER is the only known cAMP-inducible isoform of CREM expressed in T lymphocytes (76, 77). Since cAMP-mediated expression of ICER reaches extremely high levels in T cells, ICER can effectively compete and thereby repress transcription mediated by ubiquitously expressed CREB. Such competition is presumably even more efficient in T cells since they do not express potentially abundant isoforms of CREM constitutively expressed in stage specific fashion in numerous tissues of hypothalamo-pituitary-gonadal axis (100-102). Therefore, ICER competition with CREB may lead ultimately to uncoupling of CREB-mediated recruitment of CBP/p300 since ICER does not possess transactivation domain without which, at least in the context of CREB/CREM family of transcription factors, recruitment of CBP/p300 complex is unlikely. Consequently, ICER binding may lead to uncoupling of CBP, which abrogates early stages of transcriptional initiation due to the lack of CBP-associated histone-acetyl-transferase (HAT) activity resulting in the failure to maintain transcriptionally competent conformation of chromatin (103, 104). Furthermore, in the absence of CBP/p300 complex, interactions of NFAT and NFκB are likely to be affected since their full transcriptional activity seems to be dependent on interaction with CBP (105, 106). Therefore, it is possible, that CRE-like motifs adjacent to NFAT or NFκB binding motifs may, in the context of cytokine promoters, anchor CBP in order to facilitate crosstalk between Rel- and bZIP-mediated transcription.

We have previously reported that cAMP-mediated transcriptional attenuation of IL-2 expression tightly correlates with ICER induction in T cells (76, 77). In addition, we demonstrated that ICER binds DNA motifs critical for IL-2 expression either directly or indirectly via protein-protein interaction with Rel homology domain of NFAT (77). Interestingly, this interaction between bZIP and Rel homology domains, which may lead to formation of inhibitory NFAT/ICER ternary complex in the context of IL-2 promoter, is most pronounced on CD28-responsive element (CD28RE). Moreover, CD28RE has a capacity to bind other members of CREB/ATF family of transcription factors which can interact directly with CD28RE as well as with the adjacent CRE-like AP-1 motif (CD28RE-TRE) (107). In order to achieve a full activation of CD28RE resulting in recruitment of CBP/p300 complex, triggering through T cell

receptor (TcR), which leads to phosphorylation of CREB (108), must be potentiated by CD28 costimulation (109). Signal transduction pathways activated upon costimulation via CD28 are apparently required to allow CBP to integrate cooperative interactions between activated bZIP family of proteins represented by phospho-CREB and its close homologues (110) as well as Rel homology proteins such as NFAT or NFκB to orchestrate highly cooperative expression of IL-2 (110-112).

Importantly, CD28RE motif which in the context of IL-2 promoter has the highest affinity for ICER binding (77) was recently shown to be essential for conferring anergy in T lymphocytes (113). Notably, induction of the unresponsive state requires protein synthesis, suggesting that a newly synthesized protein may be required for the maintenance of the unresponsive state. Hence, ICER synthesized in response to PGE_2 as well as numerous other ligands acting through G protein coupled seven-span transmembrane receptors, could be critically involved in the induction and maintenance of anergy.

T helper cells are not homogenous population but can be subdivided on the basis of cytokine expression into at least two subsets termed T helper 1 (Th1) and T helper 2 (Th2)(114-116). Th1 secrete IL-2 and INF- , while Th2 cells produce IL-4, IL-5, IL-10, and IL-13. There is now an abundant evidence that the ratio of Th1 to Th2 cells is highly relevant to the outcome of a wide variety of syndromes including autoimmune, allergic, and infectious diseases (for review see (117-119)). Over the past ten years, numerous chemokines have been identified as attractants for different types of blood leukocytes to sites of infection and inflammation (120, 121). They are produced locally in the tissues and act on leukocytes through selective receptors. The differential expression of chemokine receptors may dictate, to a large extent, the migration and tissue homing of Th1 and Th2 cells (122, 123). It may also determine different susceptibility of Th1 and Th2 cells to human immunodeficiency virus (HIV) strains using different fusion coreceptors (124, 125). Therefore, chemokines are part of effector and amplification mechanisms of polarized Th1- and Th2-mediated immune responses and their receptors might serve as Th1 versus Th2 markers, as well as targets for selective modulation of T cell dependent immunity.

Although ICER was previously shown to downregulate IL-2 expression in transient tranfection assays (76, 77), it remained to be shown that this could be also the case *in vivo*. Analysis of ICER transgenic mice confirmed that ICER itself, rather than elevated levels of intracellular cAMP, may direct cAMP-mediated transcriptional attenuation of IL-2 expression as well as expression of numerous cytokines including certain chemokines. The results obtained herein correlate with reported observations in mice made transgenic with dominant negative mutant of CREB created by single point substitution of Ser-133 residue to Ala which cannot be phosphorylated and thus is unavailable to support CREB-mediated transcription (126), presumably due to the impaired recruitment of CBP/p300. These transgenic mice, similar to ICER transgenic mice, fail to produce IL-2 accompanied by the defect in T cell proliferation (126). Importantly, T cells of mice expressing dominant negative mutant of CREB fail to proliferate even in the presence of exogenously added IL-2, in contrast to T cells overexpressing ICER which upon addition of IL-2 are capable to partially correct the proliferative defect (data not shown). Lack of IL-2 production accompanied by defect in T cell proliferation is a hallmark of graft versus host disease resulting in the development of marked immune dysfunction (127, 128).

Importantly, ICER transgenic splenocytes in allogeneic mixed lymphocyte reaction showed lack of proliferation suggesting that ICER has capacity to suppress their ability to undergo activation.

The following procedures were used in the experiments described below.

Stable polarization of peripheral blood T cells towards type 1 or type 2 phenotype after polyclonal activation

Polarization was done according to Asselin et al (132). Briefly, PBMC from normal donors isolated from leukopak by density gradient centrifugation in Ficoll and put into culture (RPMI 1640 with L-glutamine, 5% heat-inactivated AB^+ serum (Sigma, St. Louis), penicillin, streptomycin, and sodium pyruvate. PBMC ($5x10^5$/ml) were primed with soluble sOKT3 (10ng/ml) and cultured in the presence of combination of recombinant human IL-12 (2 ng/ml) and rhIL-2 (10 ng/ml) for IL12-induced type 1 dominant phenotype, or rhIL-4 (10ng/ml) for IL-4-induced type 2 dominant phenotype for 3 days. Cells were washed and maintained with fresh cytokines for 3 to 4 more days. At the end of the first week, cell were washed and resuspended at $1x10^6$ and maintained with hrIL-2 (10 ng/ml) for one or two additional weeks, before being restimulated. Cell were restimulated with PHA (Gibco-BRL, MD) or phorbol ester (10 ng/ml) plus ionomycin (1μg/ml) for 6 hr in the absence or presence of forskolin (0.1 mM).

Preparation of human peripheral blood lymphocyte fractions

Elutriated PBLs were prepared as detailed previously (157) and briefly described below. Healthy volunteers provided informed consent to undergo leukopheresis and countercurrent centrifugal elutriation. All collection steps were performed with pyrogen-free reagents. Each donor was initially leukapheresed 5 to 7 liters whole blood on a Fenwal CS3000 blood cell separator (Baxter HealthCare Corp. Deerfield, IL) programmed for minimized neutrophil contamination. The leukopheresis concentrate was acquired in small volume collection chambers to reduce platelet contamination. This concentrate typically yielded 4 to 10 x 10^9 peripheral blood mononuclear cells, which were immediately washed in a large volume of citrate-anticoagulated normal saline to remove contaminant platelets and plasma. The washed cells were resuspended in Ca^{2+}/Mg^{2+}-free, pyrogen-free HBSS (BioWhittaker, Walkersville, MD) and elutriated using a Model J-6 M centrifuge equipped with a JE-5.0 elutriation rotor operating at 1725 x *g* and 20 ^{0}C (Beckman Instruments, Palo Alto, CA). Cells were loaded at a 120 cc/min flow rate , and then fractions were collected using stepwise flow rates ranging from 120 to 140 cc/min to obtain lymphocyte-rich fractions. Fractions were accumulated in Life Cell tissue culture vessels (Baxter HealthCare) on ice to inhibit cellular adherence. Lymphocyte fractions were further purified with density gradient centrifugation using pyrogen-free Ficoll-Hypaque (BioWhittaker) to remove red blood cells. Elutriated fractions were subjected to further separations described below then analyzed by flow cytometry analysis and immediately utilized in experiments.

Preparation of negatively selected T cells from peripheral blood lymphocytes using superparamagnetic beads

PBL subpopulations were fractioned using superparamagnetic microbeads (Miltenyi Biotec, Auburn, CA). The wash and incubation buffer was Ca^{2+}/Mg^{2+} free DPBS with 0.5% bovine serum albumin (Sigma Chemicals, Co, St. Louis) without EDTA maintained at 4 ^{0}C. 10^9 elutriated PBLs were resuspended in 4 ml of wash and incubation buffer described above and incubated first with 1 ml of cocktail containing hapten-conjugated mAbs, specific for cell surface markers expressed by non-T cell populations (anti-CD11b, CD16, CD19, CD36, and CD56) then washed and incubated

with 1 ml of anti-hapten microbeads. All incubations were carried out at 8 °C for 15 min. After the incubations were completed, the PBLs were washed and resuspended in the buffer. The indirectly labeled cells (negative selection of T cells) were applied on CS$^+$ column positioned on VarioMACS with 19G needle as a flow resistor and 30 ml wash fraction was collected. Typically, purity of negatively selected T cell population was around 95% of CD3$^+$ cells (data not shown) with prevailing representation of CD4$^+$ cells (75%) over CD8$^+$ cells (20%). After paramagnetic bead separation (Pan T) less than 1% of CD56$^+$CD16$^+$ (NK cells), CD3$^+$CD56$^+$ double positive cells expressing both T and NK cell markers, CD19$^+$ B lymphocytes, and CD14$^+$ monocytes could be detected (data not shown).

Flow Cytometry

PBLs were analyzed before and after the separations on the MACS columns using fluorescent multicolor flow cytometry (FACSort, Becton Dickinson). For cell surface analysis the following monoclonal antibodies were used: Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 and phycoerythrin (PE)-conjugated mouse anti-human CD16 purchased from PharMingen (San Diego, CA), PE-conjugated mouse anti-human CD19 from DAKO A/S (Denmark), FITC-conjugated mouse anti-human CD16 was purchased from Medarex Inc. (Annandale, NJ), PE-conjugated mouse anti-human CD56, FITC-conjugated mouse anti-human CD4, TRI color (TC)-conjugated mouse anti-human CD3, CD8, CD14 and FITC-, PE-, as well as TC- conjugated IgG subclass matched control antibodies were purchased from Caltag Laboratories (Burlingame, CA). Cells were stained at 4°C using Ca^{2+}/Mg^{2+} free DPBS with 0.5% BSA and 0.025% sodium azide as a diluent/wash FACS buffer. Non-specific FcR binding was blocked by incubation with 0.2 mg/ml human IgG (Sigma Chemical Co.) for 10-15 min. and then cells were triple-stained with FITC-, PE- and TC-conjugated Ab for 30 min. After wash with cold FACS buffer, cells were fixed in 1% paraformaldehyde in PBS. Three color analysis was then performed.

Immunoprecipitation

Cells were metabolically labeled with ^{35}S Translabel (ICN Biomedicals, CA) according to established protocols and lysed in RIPA buffer (0.15M NaCl, 50 mM Tris-Cl pH 7.2, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) supplemented with *Complete*, protease inhibitor cocktail (Boehringer Mannheim, Germany), clarified by centrifugation at 14,000 rpm for 30 min at 4 °C and precleared using Protein A Sepharose 4B beads (Pharmacia, Uppsala, Sweden). Immune complexes were collected onto Protein A Sepharose 4B beads that were pre-bound with CS4 CREM-specific antiserum, rocked for 30 min at 4 °C and followed by three washes with the RIPA buffer. Immune complexes were eluted from beads with Laemmli sample buffer and resolved by 15% SDS-PAGE under reducing conditions. The ^{35}S-signal was enhanced by PPO (2,5 diphenyloxazol, Sigma, St. Louis) treatment of the gel. ^{35}S-Labeled proteins were detected using O-XAR films (Eastman Kodak, MA) exposed for 1 to 10 days at -70 °C.

RNase Protection assay

RNA extraction was performed using RNeasy kit (Qiagen). The RNA probe for ICER was generated from pJL5 by XhoI or XbaI digestion which corresponds to full-length cDNA of human ICERII described previously (76). RNA probes hAPO3 and mAPO3 were purchased from PharMingen (San Diego, CA) and labeled with [α-^{32}P] UTP using

reagents from an RNA probe kit (Ambion). These probes were used for RNase protection studies according to the protocol provided by Ambion (RPAII Ribonuclease Protection Assay kit).

Production and characterization of ICER transgenic mice

A 0.36 kb fragment encompassing the ICER coding sequence driven by the proximal lck promoter was introduced in the germline of mice by pronuclear microinjection (158). Out of several independent founder lines generated, three were selected for analysis based on the level of expression of the transgene. For measurements of lymphocyte proliferation, freshly isolated lymphocytes (2x105) were cultured in triplicate in 200 µl DMEM medium with 10% FCS (GIBCO-BRL) in 96 well tissue culture plates (Falcon). Lymphocytes were activated for 48 hours at 37 oC by treatment with PMA (10 ng/ml) and ionomycin (1 µg/ml), the anti-CD3 mAb, 145.2C11 (10 µg/ml immobilized on plastic tissue culture plates), or ConA (2 µg/ml). 48 hours after activation cells were labeled for 18 h by incubation in 3H-thymidine-containing tissue culture medium (1 µCi/ml; specific activity 2 Ci/mmol) (Amersham). Cells were collected onto glass fibre filter mats and 3H-thymidine incorporation was measured in scintilation counter. For measurement of allostimulation, splenocytes from ICER transgenic or nontransgenic mice were cocultivated either with syngeneic splenocytes from CB57BL/6 mice or allogeneic splenocytes from BALB/c for 48 hrs then labeled and counted as described above.

Example 18

Human Peripheral Blood T Lymphocytes are Potent Inducers of ICER Protein

ICER mRNA can be induced by cAMP elevating agonists (e.g. forskolin or PGE2) in developing as well as mature T lymphocytes (76). To ascertain whether ICER mRNA is efficiently translated, the presence of ICER protein was monitored by immunoprecipitation of the lysate prepared from human peripheral blood T lymphocytes at different time points following treatment with forskolin or PGE2 (Fig. 13). Immunoprecipitations of the whole cell extracts confirmed stability of ICER protein, its efficient labeling and continuous accumulation in the cell depending on elapsed time after the treatment. These observations are strikingly different from our findings in human medullary thymocytes, where induction of ICER protein after forskolin treatment is only transient (after 3 hours) with lack of any detectable ICER-specific signal following 12 hours of forskolin treatment (77). Surprisingly, ICER expression in mature T lymphocytes is remarkably stable during first 18 hours after forskolin or PGE2 treatment (Fig. 13). Our findings based on immunoprecipitation of ICER protein correlate with RNase protection assays using several different ICER and CREM probes (76) which indicate that all detected ICER isoforms in T lymphocytes utilize cAMP-inducible P2 promoter of CREM gene used for transcription by ICER only (76, 77,97). Thus, ICER and ICER-g proteins (ICERI, ICERII, ICERIγ, and ICERIIγ) are the only cAMP inducible members of CREM family detectable in human peripheral blood T lymphocytes (Fig. 13). Remarkably, ICER can be readily induced in peripheral blood T lymphocytes during isolation most likely through release of numerous cAMP-elevating agonists (data not shown). Nevertheless, combination of elutriation and negative selection allowed us to isolate 'untouched' peripheral blood T lymphocytes without elevated levels of ICER prior to forskolin stimulation (Fig. 13). Indeed, in untreated T lymphocytes isolated by

negative selection (see below), immunoprecipitations of the whole cell lysates with antiserum raised against full length CREM (CREMα) (129) failed to detect constitutively expressed ICER or CREM proteins prior to forskolin treatment (see Fig. 13, lane 1 for CREM-specific antiserum denoted C versus normal rabbit control antiserum N in lane 2). In contrast, after three hours of forskolin or PGE_2 treatment (Fig. 1, lane 3 and lane 9, respectively) distinct signals for both ICERI and ICERII (ICER) as well as their counterparts missing exon-γ, ICERI-γ and ICERII-γ (ICER-γ) could be detected. Accumulation of ICER proteins after forskolin and PGE_2 treatments continued progressively over the time reaching extremely high levels first after forskolin (at 12 hrs) and then after PGE_2 treatment (at 18hrs). Our data indicate that physiologically significant amounts of ICER can be readily induced in fresh human peripheral blood T lymphocytes in response to relatively unstable ligands such as PGE_2 suggesting that even transient activation of signal transduction pathway(s) may relay signals sufficient for dramatic upregulation ICER.

Example 19

Stable Polarization of Peripheral Blood T Cells Towards T helper -1 and T helper -2 Phenotypes

Polarization of T helper effector functions, implicated in numerous diseases, represents potentially powerful intervention for directing immune responses via an adoptive therapy. In the attempt to elucidate molecular mechanism underlying cAMP-mediated inhibition of T helper effector functions, *in vitro* conditions to drive resting polyclonal human T cells towards stable T helper-1 (Th1) or T helper-2 (Th2) phenotypes according to Asselin et al. 1997 (130) have been established. Unselected peripheral blood mononuclear cells from normal donors primed with soluble anti-CD3 monoclonal antibody (sOKT3) in the presence of human recombinant cytokines (IL-12 and IL-2 for Th1 dominant subset, and IL-4 for Th2 dominant subset) (for details see below) were later expanded in the presence of IL-2. To compare expression of cell surface markers between Th1 and Th2 populations, flow cytometry analysis was performed prior to PHA-restimulation. This analysis revealed only relatively minor alterations in CD4/CD8 ratios during *in vitro* polarization of T cells and confirmed tendency towards memory phenotype represented by major shift towards cells harboring $CD45RO^+$ marker in both populations (Fig. 14A). Thus, cytokine and chemokine expression following restimulation originates most likely from memory T cells judging from acquisition of their ability in both Th1 and Th2 subsets to produce INF-γ (Fig. 14B) which is a fundamental property of memory T cells (131).

Example 20

Cyclic-AMP-mediated Transcriptional Attenuation of Cytokines and Chemokines Expressed in Th1 and Th2 Dominant Subsets Correlates with Induction of ICER

As demonstrated above, ICER has a capacity to interact with NFAT in the context of CD28RE of IL-2 promoter either by direct protein-protein interaction or indirectly via DNA binding to the adjacent AP-1 motif (77). The notion, that ICER may form inactive NFAT/ICER complex which could lead to inhibition of NFAT-driven cytokines such as IL-2 has been previously tested in transient transfection assays (76, 77). These experiments, which monitored in activated Jurkat T cells effects of ectopically expressed ICER on transcription of NFAT-driven cytokine promoters linked to CAT or Luc reporters, suggested that ICER might be responsible for cAMP-mediated transcriptional

attenuation of numerous NFAT-driven cytokines. To prove that ICER is also able to inhibit endogenous expression of cytokines in activated human T lymphocytes, human peripheral blood T cells were polarized towards Th1 and Th2 phenotypes (132) and then restimulated with PHA in the absence or presence of forskolin (Fig. 14B). RNase protection analysis revealed that, both Th1 and Th2 dominant subsets expressed INF--γ, while Th2 dominant population expressed significantly higher levels of IL-4 and IL-5 cytokines, a hallmark of Th2 phenotype (Fig. 14). Furthermore, cAMP-mediated inhibition of endogenously expressed cytokines characteristic for Th1 and Th2 phenotypes tightly correlates with forskolin-mediated induction of ICER in both subsets suggesting that ICER could be responsible for observed inhibitory effect in cAMP-mediated transcriptional attenuation of cytokine expression. Moreover, early expression of chemokines which was found to be T helper-specific is also susceptible to cAMP-mediated inhibition for both MIP1α and MIP1β but not RANTES, and correlates inversely with ICER induction (Fig. 14C).

Example 21

Cyclic-AMP-mediated Downregulation of Cytokine Expression in T cells with Th1 and Th2 Dominant Phenotypes is Critically Dependent on Restimulation

Susceptibility or resistance of cytokine expression to cAMP-mediated inhibition in Th1 and Th2 dominant T cell subsets is critically dependent upon restimulation most remarkably reflected by IL-2 expression. Defect in IL-2 expression upon the failure to deliver costimulatory signals is believed to be crucial for induction and maintenance of anergy (133). Indeed, delivery of both signals often mimicked by phorbol ester and ionomycin treatments, leads to expression of supraphysiological levels of IL-2 in both Th1 and Th2 dominant populations which is likely to promote resistance of cytokine expression to cAMP-mediated inhibition by forskolin (Fig. 15). In contrast, mitogenic stimulation via phytohemaglutinin (PHA) which relays signals mainly through TcR, induces in the absence of costimulatory signal(s) significantly lower levels of IL-2 which correlates with susceptibility to cAMP-mediated transcriptional attenuation (Fig. 15). This apparent disparity in capacity of cAMP to mediate inhibition of cytokine expression in the absence of IL-2 was previously noted in PGE_2-mediated IL-4 and IL-5 inhibition (134). It was proposed that physiological differences which distinguish productive proliferation from anergy in T lymphocytes are best characterized by presence or absence of IL-2-mediated autocrine loop which may also reflect the differential susceptibility of these cells to the suppression from outer enviromental stimuli (135). Therefore, signals which make T cells to proliferate and produce high levels of IL-2 are also likely to make them resistant to cAMP-mediated inhibition in contrast to the cells with low or significantly reduced IL-2 expression. Indeed, our data support the notion that in the absence of costimulation or presence of suboptimal costimulation which leads to low levels of IL-2 expression, both Th1 as well as Th2 cells could be deprived of T helper functions more easily in comparison with the situation when costimulation leads to vigorous IL-2 expression. However, both treatments used for restimulation (phorbol ester plus ionomycin, as well as PHA) are effective for expression of other NFAT-driven cytokines such as IL-4, IL-5, and IL-13 which are required for effector functions of Th2 phenotype. Importantly, only PHA restimulation, which failed to

induce vigorous IL-2 expression, rendered IL-4, IL-5, and IL-13 expression susceptible to cAMP-mediated inhibition (Fig. 15).

Unlike in rodents, where differential levels of methylation of INF- promoter reflect differential expression of INF-γ in Th1 versus Th2 subsets (136), in humans, indiscriminate expression of INF-γ after restimulation in both T helper subsets seems to correlate with higher overall INF-γ expression reported to be due to uniform hypomethylation of INF-γ promoter in both subsets (for review see (137)). Nevertheless, after PHA-mediated restimulation Th2 cells are more susceptible to cAMP-mediated inhibition of INF-γ expression in contrast to Th1 cells which retain at least partially their capacity to maintain INF-γ expression even in the presence of forskolin. Since number of motifs involved in expression of INF-γ are hypomethylated in both subsets, observed differential susceptibility to cAMP-mediated inhibition might be related to the p38MAP kinase signaling pathway which was reported to be relevant for INF-γ expression in Th1 but not Th2 cells (137).

Example 22

Defective Production of IL-2 and INF-γ as well as MIP-1α and MIP-1β Accompanied by Impaired T Cell Proliferation in Transgenic Mice Expressing ICER

To prove that ICER per se, could be responsible for observed cAMP-mediated transcriptional attenuation of cytokine and chemokine expression independently on forskolin-mediated increase of intracellular cAMP, we generated ICER transgenic mice. ICER transgenic mice, expressing human homologue of ICERII isoform preferentially in lymphoid cells under the control of proximal *lck* promoter, sustains high levels of ICER expression in transgenic thymus as confirmed by immunoprecipitations using CREM-specific antiserum (Fig. 17A). Thus, high levels of ICER expressed under control of heterologous promoter in transgenic thymus allowed us to directly test inhibitory role of ICER on IL-2 and INF-γ expression as well as expression of chemokines expressed early in activated transgenic thymocytes. In contrast to thymocytes from nontransgenic littermates which expressed normal amounts of IL-2 and INF-γ when activated with phorbol ester plus ionomycin, transgenic thymocytes failed to express IL-2 and INF-γ after activation (Fig. 16A). Moreover, ICER transgenic mice failed to express MIP-1α and MIP-1β as well as lymphotactin (Ltn) and IP-10 while expression of RANTES, although modest, was almost unaffected suggesting high specificity of ICER-mediated inhibition in both induced as well as background cytokine or chemokine expression (Fig. 16B).

The ability of ICER expression to alter development or effector functions of transgenic lymphocytes was investigated. The effects of ICER on differentiation of cells with constitutive ICER expression in the lymphoid compartment of the mice were determined. Analysis of transgenic animals revealed that total numbers of thymocytes and splenocytes are equivalent in ICER transgenic as well as nontransgenic control littermates (Fig. 17D). Both transgenic and nontransgenic thymocytes expressed normal levels of CD3 and TcR a/b, and there were normal numbers of double-negative ($CD4^-/CD8^-$), double positive ($CD4^+/CD8^+$) and single positive ($CD4^+$ and $CD8^+$) thymocytes and splenic T cells in transgenic animals (data not shown). Thus, ICER expression did not noticeably disrupt T cell development. In contrast, ICER transgenic splenocytes and peripheral blood lymphocytes displayed various extent of proliferation defect following activation using ConA, immobilized anti-CD3 monoclonal antibody 2C11 or phorbol ester

plus ionomycin treatment (Fig. 17B). Importantly, the most dramatic differences were seen after mitogenic ConA stimulation, while phorbol ester plus ionomycin treatment exhibited significant yet not as striking differences in proliferation in spleen of ICER transgenic mice (Fig. 17B).

Our observations in ICER transgenic mice correlate with susceptibility of mitogenic stimulation to cAMP-mediated inhibition in polarized human peripheral blood T lymphocytes where PHA stimulated cytokine and chemokine expression is susceptible to forskolin-mediated inhibition while phorbol ester plus ionomycin treatment renders cells resistant to inhibitory effects of forskolin (Fig. 15). Importantly, these data tightly correlate with lack of IL-2 expression analyzed by RNase protection in ICER transgenic thymocytes (Fig. 16) as well as in polarized human peripheral blood T lymphocytes restimulated by PHA (Fig. 14), suggesting that ICER may play an important role in cAMP-mediated inhibition of cytokine and chemokine expression through modulation of IL-2 expression. Moreover, allogeneic stimulation in mixed lymphocyte reaction using splenocytes from ICER transgenic or nontransgenic mice cocultivated with either syngeneic splenocytes from CB57BL/6 mice or allogeneic splenocytes from BALB/c mice yielded dramatically different thymidine uptake suggesting that ICER transgenic splenocytes are phenotypically and functionally distinct from nontransgenic splenocytes (Fig. 17C). Although the molecular mechanism of ICER action effectively aborts T cell clonal expansion *in vitro*, it does not seem to result in clonal elimination of the responding population. The failure to generate effective proliferation suggests that ICER transgenic lymphocytes are functionally inactivated.

Although ICER is capable of binding to numerous NFAT/AP-1 motifs essential for cytokine expression *in vitro*, it remained to be shown that this binding is also reflected by transcriptional attenuation of the endogenous cytokine expression *in vivo*. Our findings in ICER transgenic mice tightly correlate with previously reported observations in mice made transgenic with dominant negative mutant of CREB which due to the substitution of Ser133 residue to Ala cannot be phosphorylated and thus unable to produce IL-2 which is accompanied by severe defect in T cell proliferation (126), presumably due to its impaired ability to recruit transcriptional integrator CBP/p300. Indeed, CBP-deficient mice show general proliferation defect (96) supporting the notion that ICER (or dominant negative mutant of CREB) can compete with endogenously expressed CREB and thus abrogate recruitment of CBP/p300.

In contrast to artificial nature of dominant negative CREB mutant, ICER is the only known cAMP-inducible transcriptional repressor which is in T lymphocytes physiologically relevant (76, 77). Furthermore, induction of ICER tightly correlates with cAMP-mediated transcriptional attenuation of IL-2 expression, usually accompanied by lack of T cell proliferation. Although it is unlikely that ICER alone can effectively compete with activated complex containing phospho-CREB associated with CBP/p300, it is possible that after disintegration of the complex in the absence of IL-2, ICER might be able to compete with CREB alone, thus undermining transcriptional competence for next round of transcriptional activation. It is clear, however, that it is the failure to produce the IL-2 that is the most important determinant of whether anergy is induced (113, 133). Thus, ICER binding to CD28RE of IL-2 promoter, followed by TcR-mediated translocation of NFAT, may lead to formation of inactive NFAT/ICER complex unable to recruit

CBP/p300 which could make CD28RE motif unresponsive resulting in the lack of IL-2 expression. Moreover, ICER induction in response to PGE_2, may lead in the absence of IL-2 production to reported PGE_2-mediated suppression of T cell effector functions (77, 134).

Since one of the primary functions of the TcR in the presence of costimulatory signals is to initiate the IL-2-mediated autocrine loop responsible for completing the activation of proliferation it is possible that IL-2 receptor-mediated activation of PI3-kinase (139, 140) may relay signals required for activation of $p70^{rsk}$ kinases which are responsible for growth factor-mediated phosphorylation of CREB (98, 141, 142). Subsequent transcriptional induction is thought to proceed via the CBP/p300-dependent recruitment of RNA polymerase II complexes. Thus, IL-2-mediated autocrine loop induced by phorbol ester and ionophore may render T cells resistant to cAMP-mediated inhibition even in the presence of ICER due to yet unidentified modification(s) of ICER and/or CBP/p300 activatory complex which, once assembled, might be unrestrained by actions of ICER, in contrast to the situation prior to recruitment of CBP when ICER on CRE sites could be sufficient for effective competition with CREB (77, 80). Indeed ICER-mediated defect in T cell proliferation can be at least in part restored by the exogenously added IL-2 (data not shown) in striking contrast to the inability of IL-2 to rescue T cell proliferation in cells made transgenic by dominant negative mutant of CREB (126).

Despite similarities between both transgenic models, one major difference stems from the fact that ICER is likely to be tightly regulated due to the cyclical nature of its expression which corresponds to actual physiological conditions in the cell (143). This is in striking contrast to the dominant negative mutant of CREB which is extremely stable irrespective of physiological conditions in the cell due to the inherently stable properties of ubiquitously expressed wild type CREB (80). Therefore, ICER-mediated conditional defect of T cell proliferation seems to be critically dependent on nature of restimulation reflecting differential activation of numerous signal transduction pathways involved in T cell costimulation in ICER transgenic mice. Moreover, conditional character of ICER-mediated defect in T cell proliferation suggests that ICER may represent tightly controlled molecular target regulated by sophisticated network of signal transduction pathways relaying their effects through postranslational modifications affecting ICER's capacity to function. One reason for tight scrutiny might be due to ICER's ability to bind CD28RE DNA binding motifs critical for conferring anergy in the context of IL-2 promoter (77, 113). Thus, ICER binding may abrogate IL-2 production and lead to subsequent disruption of IL-2-mediated autocrine loop which could be critical for further modifications of CBP/p300 complex responsible in T cells for progression through cell cycle (144-148). Moreover, modifications of CBP/p300 complex in the presence of IL-2 may render T cells resistant to cAMP-mediated inhibition of cytokines in striking contrast to their susceptibility in the absence of IL-2 production.

It is well established that activatory complexes, containing NFAT or NFkB Rel homology proteins are playing dominant role in the transcriptional regulation of numerous cytokine and chemokine promoters (149, 150). NFAT and NFκB are often associated with bZIP transcriptional factors such as CREB, Fos, and Jun which in turn are responsible for recruitment of CBP/p300 (86, 151). Hence, consequences of ICER actions could be either direct, for example by binding to unoccupied CD28RE motifs in the promoter context of critical cytokines and chemokines such as IL-2 (77)

and RANTES (152), or indirect, by downregulation of Fos and Jun expression through CRE-like motifs located in their respective promoters (98, 153, 154). Thus, there is a variety of direct as well as indirect mechanisms which might conditionally implicate ICER in transcriptional attenuation of numerous cytokines and chemokines.

In the course of our work, we have noticed that ICER transgenic lymphocytes had an enhanced instability in T cell proliferation defect observed independently on nature of costimulation. One possible explanation might be induction of endogenous expression of activatory leucine zipper family member(s) which might be responsible for amelioration of ICER-mediated defect. Similar situation, in the case of CREB deficient mice, leads reportedly to expression of activatory CREM isoform(s), which over the time, completely compensated for observed defects (155). However, in the case of ICER transgenic mice we did not detect any significant compensatory expression of CREM isoforms perhaps due to inability of CREM-specific P1 promoter to function in T lymphocytes (data not shown). Nevertheless, there are still a large number of different CREB/ATF isoforms, including many close homologues containing bZIP domain associated with active transactivation domain, which may ameliorate ICER-mediated defect by compensatory expression. Therefore combination of ICER heterodimerization with compensatory expression of activatory isoforms may create a wide spectrum of conditions allowing partial or even complete compensation of ICER-mediated inhibition.

In our attempt to elucidate the role of ICER in cAMP-mediated suppression of cytokine and chemokine expression, we analyzed correlation between cAMP-mediated inhibition and ICER induction in *in vitro* polarized human peripheral blood T lymphocytes. Our analysis in human polarized peripheral blood T lymphocytes with Th1 or Th2 phenotypes revealed critical role of IL-2 expression for ability of T cells to be either resistant or susceptible to cAMP-mediated inhibition of cytokine and chemokine expression. Critical importance of costimulation, underlined by induction of potent transcriptional repressor ICER which has capacity to inhibit IL-2 expression itself, may lead to modulation of sophisticated network controlling diverse effector functions of T cells upon activation such as clonal expansion, induction of anergy, or clonal deletion. Therefore, it is possible that temporal constraints on ICER induction circumvented in ICER-transgenic lymphocytes by constitutive expression of ICER prior to T cell activation, may deprive T cells of their ability to induce essential components of the transcriptional machinery (such as AP-1 complex) which are believed to be necessary in assembly of transcriptional complexes by CBP/p300. Moreover, ICER's ability to mask critical CD28RE motifs may lead to failure to recruit CBP/p300 which would affect highly cooperative interactions on IL-2 promoter leading to subsequent disruption of IL-2-mediated autocrine loop. Absence of IL-2 production results then in abrogation of cytokine production associated with defect in T cell proliferation as observed in ICER transgenic mice.

However, conditional character of this defect seems to be critically dependent on the nature of restimulation, thus underscoring importance of costimulatory signal transduction pathway(s) mimicked by tumor promoters such as phorbol ester. Phorbol ester, previously reported to be able to rescue T lymphocytes from anergy presumably via release of Ras blockade (156), is believed to interfere with induction and/or activity of AP-1 complex (152). Therefore, ICER transgenic lymphocytes constitutively expressing ICER from heterologous promoter, might at

least in part function through their enhanced capacity to generate functional AP-1 complex (85) thought to be instrumental for highly cooperative assembly of transcriptional factors on IL-2 promoter directed by costimulation (149). It is tempting to speculate that, while AP-1 is absent, ICER could bind vacant CRE-like DNA-binding sites instead of AP-1 complex and in parallel inhibit expression of AP-1 components. Since ICER lacks transactivation domain, competition with ubiquitously expressed CREB or Jun family members may prevent recruitment of CBP/p300 leading in the absence of CBP-associated HAT activity to transcriptional silencing of the relevant promoter(s). Therefore, ICER binding leading directly or indirectly to impaired function of AP-1 via transcriptional attenuation of Fos and Jun (10) may favor ICER-mediated cooperative interaction with Rel homology domain of NFAT as demonstrated *in vitro* by formation of inhibitory NFAT/ICER complex (77).

Although, these events are likely to be mutually interdependent, they are likely to reflect conditional and temporal character of cAMP-mediated inhibition as well as its potential reversibility. Thus nature of costimulation represents clearly a decisive moment for shaping proliferative response dictating either susceptibility or resistance to enviromental inhibitory stimuli modulated by ICER-mediated transcriptional attenuation. It is also conceivable that costimulation signal derived by phorbol ester, a known tumor promoting agent, may escape ICER-mediated inhibition since it activates events associated with tumorigenesis.

IV. Differential Inducibility of ICER and its Role in Modulation of Fas Ligand Expression in T and NK Lymphocytes

Under some conditions the engagement of antigen receptor initiates apoptosis of T lymphocytes through the induced expression of Fas ligand. Recent reports indicate that forskolin, an activator of cAMP/PKA pathway, results in antagonism of Fas-dependent, activation-induced cell death by suppressed expression of Fas ligand. Here we report that forskolin-mediated induction of ICER (inducible cAMP early repressor) correlates with transcriptional attenuation of Fas ligand expression in the activation-induced cell death model 2B4 T cell hybridoma as well as human peripheral blood T lymphocytes. Remarkably, ICER is expressed prior to forskolin treatment in a freshly isolated subset of human peripheral blood NK cells enriched for $CD56^+$ cells, whereas in peripheral blood $CD3^+$ T cells ICER is inducible and in $CD19^+$ B cells its expression appears to be refractory to forskolin treatment. In both T and NK lymphocytes increased expression of ICER correlated with decreased Fas ligand expression. We show that ICER binds specifically to the proximal NFAT DNA binding site of the Fas ligand promoter represented by one of the two NFAT motifs essential for NFAT-mediated Fas ligand expression. In the presence of the minimal NFAT DNA-binding domain the proximal NFAT motif allows ICER and NFAT to form a NFAT/ICER ternary complex *in vitro*. Moreover, in the activated 2B4 T cell hybridoma the proximal NFAT motif participates in the down-regulation of the Fas ligand promoter mediated by ICER. These findings provide further insight into the mechanism involved in cAMP-mediated transcriptional attenuation of Fas ligand expression in human T and NK lymphocytes.

T cell receptor (TcR)-mediated activation of T-cells can induce programmed cell death by a Fas-dependent pathway (159). The T cell hybridoma 2B4.11, one model to study cell death in T lymphocytes, undergoes apoptosis when it is stimulated by its cognate antigen, monoclonal antibody to CD3ε, or phorbol ester-ionomycin treatment (160, 161). Recent reports indicate that forskolin, an activator of the cAMP/PKA pathway, results in antagonism of

Fas-dependent cell death regardless of the presence or absence of transgenic Bcl-2 (162). Furthermore it was shown that forskolin not only inhibits activation-induced cell death (AICD) but also suppresses expression of Fas ligand (FasL) (162) which has been identified and cloned (163) including its promoter region (164). FasL is a type II transmembrane protein that is a member of the TNF superfamily. FasL-deficient *gld* mice and Fas-deficient *lpr* mice with generalized lymphoproliferative disorder have a defect in antigen-stimulated apoptosis, strongly supporting the notion that Fas is involved in activation induced T cell apoptosis (165).

It is likely that some of the signaling molecules and transcription factors that regulate T cell proliferation and effector functions are also important regulators of genes controlling commitment to apoptosis.

cAMP signaling is inhibitory to T cell proliferation and effector functions (166). The diterpene forskolin is a known activator of cAMP/PKA signaling pathway acting via direct stimulation of adenylyl cyclase. ICER can be induced in T lymphocytes in response to forskolin-mediated elevation of intracellular cAMP by the alternative utilization of an internal promoter in CREM gene (167, 168). ICER is a potent transcriptional repressor of cAMP-responsive gene transcription that appears to serve as a negative regulator of the CREB and CREM family of transcription factors as well as other related bZIP family members. The importance of CREB in fetal T cell development was recently demonstrated by impaired development of the $\alpha\beta$ but not $\gamma\delta$, lineage in CREB deficient mice (169). In contrast, a dominant-negative form of CREB which is a functional homologue of ICER had unperturbed T cell development when expressed under a CD2 promoter but exerted a defect in thymocyte proliferation and IL-2 production (170). The ICER isoforms represent a unique cAMP-inducible CREM subfamily of transcription repressors containing cAMP-response elements within its own internal P2 promoter. Due to autoregulation of the cAMP-inducible P2 promoter, the expression of transcriptional repressor ICER can be intrinsically rhythmical. The rhythmical expression of ICER was first described in the pineal gland and in the hypothalamic-pituitary gonadal axis (171, 172). However, the P2 promoter of ICER is also differentially inducible in organs other than the pineal and hypothalamic-pituitary gonadal axis such as specific subsets of human lymphocytes (167, 168). Thus ICER by its differential expression in response to cAMP/PKA signaling in lymphocytes could be a candidate for regulating molecules such as FasL.

The transcription factor, NFAT (nulear factor of activated T cells), which is instrumental for the inducible expression of many cytokine genes, also plays a critical role in the regulation of TcR-mediated FasL expression (173, 174). Two sites within FasL promoter, proximal and distal, identified through DNase I footprinting (173), bind NFAT proteins from activated T cells. Although both sites appear important for optimal expression of FasL in activated T cells, these sites do not seem to be required for constitutive FasL expression in Sertoli cells suggesting that inducible and constitutive FasL expression could be regulated differently. Nevertheless, a failure to induce FasL expression accompanied by splenomegaly and lymphoproliferative disorder of T cells in NFATp-deficient mice implicated NFATp as a possibly critical transcription factor involved in Fas-mediated apoptosis of activated T cells (175). Moreover, the proximal NFAT motif of the FasL promoter could associate with ICER in a fashion similar to that of the proximal NFAT motif of IL-2 promoter (168), as well as numerous NFAT/AP-1 composite DNA binding sites previously identified as

being essential in the context of IL-2, GM-CSF, IL-4 and TNFα promoters (168, 176-179). In the presence of the minimal NFAT DNA-binding domain (NFAT-DBD) (22, 23) the proximal NFAT motif of the FasL promoter could form NFAT/ICER ternary complexes in a fashion similar to those of the cytokine NFAT DNA binding sites interacting with ICER either by protein-protein interactions via NFAT DBD, or by direct protein-DNA interactions via ICER. These considerations are consistent with, and further support, a hypothesis that ICER may act to regulate FasL expression. Furthermore, in transient transfection assays forskolin-mediated induction of ICER correlates closely with the ability of ectopically expressed ICER to repress activation of the FasL promoter as well as other NFAT-driven cytokine promoters such as IL-2, GM-CSF, and TNF (168).

To study the role of ICER in cAMP-mediated transcriptional attenuation of FasL expression the differential expression of ICER in lymphocytes was assessed and the time-course of forskolin-mediated down-regulation of FasL expression accompanied by ICER induction in activated 2B4 T hybridoma cells was monitored. In addition, the transcriptional attenuation of FasL expression was compared with ICER induction in activated human peripheral blood T lymphocytes in the presence of forskolin. The results of our analysis in T lymphocytes indicate that forskolin-mediated induction of ICER expression correlates with a down-regulation of FasL expression. In contrast, human peripheral blood NK lymphocytes under conditions used in our study, exhibit pre-existing levels of ICER prior to forskolin stimulation both on the mRNA and protein levels. However, transcriptional de-repression of FasL expression, reflected by cessation of ICER expression in phorbol ester activated NK lymphocytes, could implicate ICER in the modulation of FasL expression in a direct as well as an indirect fashion.

The following techniques and procedures were used in the experiments below.

Preparation of human peripheral blood lymphocyte fractions

Elutriated PBLs were prepared as detailed previously (182) and briefly described below. Healthy volunteers provided informed consent to undergo leukapheresis and countercurrent centrifugal elutriation. All collection steps were performed with pyrogen-free reagents. Each donor was initially leukopheresed 5 to 7 liters whole blood on a Fenwal CS3000 blood cell separator (Baxter HealthCare Corp. Deerfield, IL) programmed for minimized neutrophil contamination. The leukopheresis concentrate was acquired in small volume collection chambers to reduce platelet contamination. This concentrate typically yielded 4 to 10 x 10^9 peripheral blood mononuclear cells, which were immediately washed in a large volume of citrate-anticoagulated normal saline to remove contaminant platelets and plasma. The washed cells were resuspended in Ca^{2+}/Mg^{2+}-free, pyrogen-free HBSS (BioWhittaker, Walkersville, MD) and elutriated using a Model J-6 M centrifuge equipped with a JE-5.0 elutriation rotor operating at 1725 x *g* and 20 ^{0}C (Beckman Instruments, Palo Alto, CA) (183, 184). Cells were loaded at a 120 cc/min flow rate , and then fractions were collected using stepwise flow rates ranging from 120 to 140 cc/min to obtain lymphocyte-rich fractions. Fractions were accumulated in Life Cell tissue culture vessels (Baxter HealthCare) on ice to inhibit cellular adherence. Lymphocyte fractions were further purified with density gradient centrifugation using pyrogen-free Ficoll-Hypaque (BioWhittaker) to remove red blood cells. Elutriated fractions were subjected to further separations described below then analyzed by flow cytometry analysis and immediately utilized in experiments.

Preparation of CD3$^+$, CD19$^+$,CD56$^+$ and negatively selected T cells from peripheral blood lymphocytes using superparamagnetic beads

PBL subpopulations were fractioned using superparamagnetic microbeads (Miltenyi Biotec, Auburn, CA) preconjugated with mouse anti-human mAbs to either CD3, CD19 or CD56 for positively selected cells, while negatively selected T cells were prepared by using a cocktail of hapten-bound mAbs with specifity for cell surface markers expressed by non-T cell populations (anti-CD11b, CD16, CD19, CD36, and CD56), with subsequent incubation of superparamagnetic anti-hapten microbeads. The wash and incubation buffer was Ca^{2+}/Mg^{2+} free DPBS with 0.5% bovine serum albumin (Sigma Chemicals, Co, St. Louis) without EDTA maintained at 4 ^{0}C. For preparation of positively selected cells, fresh PBLs were washed and resuspended at 1 x 10^8 cells per 0.8 ml to which 0.2 ml of antibody coated microbeads was added. For preparation of negatively selected T cells, 10^9 elutriated PBLs were resuspended in 4 ml of wash and incubation buffer described above and incubated first with 1 ml of cocktail containing hapten-conjugated mAbs, then washed and incubated with 1 ml of anti-hapten microbeads. All incubations were carried out at 8 ^{0}C for 15 min. After the incubations were completed, the PBLs were washed and resuspended in the buffer. The directly labeled cells (CD3$^+$, CD19$^+$, or CD56$^+$) were applied on VS$^+$ columns positioned on MidiMACS magnet (2.5 x 10^8 cells per column). After four 3 ml washes, columns were removed from the magnets and positively selected cells were flushed out with 5 ml of the buffer per column. Cells were evaluated for expression of CD3, CD19, and CD56 cell surface molecules by multicolor flow cytometry. Typically, purity of CD3$^+$ T cell, CD19$^+$ B cell, and CD56$^+$ NK cell populations was higher than 95% (data not shown) with significant (about 10%) representation of CD3$^+$CD56$^+$ double positive cells expressing both T and NK cell markers in the case of CD3$^+$ T cells and CD56$^+$ NK cells. In these cell populations less than 1% of CD14$^+$ monocytes or CD19$^+$ B cells could be detected (data not shown). The indirectly labeled cells (negative selection of T cells) were applied on CS$^+$ column positioned on VarioMACS with 19G needle as a flow resistor and 30 ml wash fraction was collected. Typically, purity of negatively selected T cell population was around 90% of CD3$^+$ cells (data not shown) with prevailing representation of CD4$^+$ cells (80%) over CD8$^+$ cells (10%). After paramagnetic bead separation (Pan T) less than 1% of CD56$^+$CD16$^+$ (NK cells), CD3$^+$CD56$^+$ double positive cells expressing both T and NK cell markers, CD19$^+$ B lymphocytes, and CD14$^+$ monocytes could be detected (data not shown).

Flow Cytometry

PBLs were analyzed before and after the separations on the MACS columns using fluorescent multicolor flow cytometry (FACSort, Becton Dickinson). For cell surface analysis the following monoclonal antibodies were used: Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 and phycoerythrin (PE)-conjugated mouse anti-human CD16 purchased from PharMingen (San Diego, CA), PE-conjugated mouse anti-human CD19 from DAKO A/S (Denmark), FITC-conjugated mouse anti-human CD16 was purchased from Medarex Inc. (Annandale, NJ), PE-conjugated mouse anti-human CD56, FITC-conjugated mouse anti-human CD4, TRI color (TC)-conjugated mouse anti-human CD3, CD8, CD14 and FITC-, PE-, as well as TC- conjugated IgG subclass matched control antibodies were purchased from Caltag Laboratories (Burlingame, CA). Cells were stained at 4^{0}C using Ca^{2+}/Mg^{2+} free DPBS with

0.5% BSA and 0.025% sodium azide as a diluent/wash FACS buffer. Non-specific FcR binding was blocked by incubation with 0.2 mg/ml human IgG (Sigma Chemical Co.) for 10-15 min. and then cells were triple-stained with FITC-, PE- and TC-conjugated Ab for 30 min. After wash with cold FACS buffer, cells were fixed in 1% paraformaldehyde in PBS. Three color analysis was then performed.

Cell lines

The NK3.3 human NK cell line (gift from Dr. J. Kornbluth) was maintained in RPMI 1640 with 25 mM Hepes, 15% heat inactivated fetal bovine serum, 15% lymphocult T (Biotest Diagnostics) without antibiotics supplemented with IL-2 (15U per ml) (185). NK92 human NK cell line (gift from Dr. E. Long) were maintained in Myelocult H5100 (StemCell Technologies, Vancouver) in the presence of 100U IL-2 per ml (186). 2B4.11 T cell hybridoma (gift from Dr. J. Ashwell) was grown exponentially in RPMI 1640 (for luciferase assay purposes without phenol red) with 10 % heat inactivated fetal bovine serum, penicillin (Gibco, BRL), and gentamicin (Gibco, BRL).

Immunoprecipitation

Cells were metabolically labeled with ^{35}S Translabel (ICN Biomedicals, CA) according to established protocols and lysed in RIPA buffer (0.15M NaCl, 50 mM Tris-Cl pH 7.2, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) supplemented with *Complete*, protease inhibitor cocktail (Boehringer Mannheim, Germany), clarified by centrifugation at 14,000 rpm for 30 min at 4 °C and precleared using Protein A Sepharose 4B beads (Pharmacia, Uppsala, Sweden). Immune complexes were collected onto Protein A Sepharose 4B beads that were pre-bound with CS4 CREM-specific antiserum, rocked for 30 min at 4 °C and followed by three washes with the RIPA buffer. Immune complexes were eluted from beads with Laemmli sample buffer and resolved by 15% SDS-PAGE under reduced conditions. The ^{35}S-signal was enhanced by PPO (2,5 diphenyloxazol, Sigma, St. Louis) treatment of the gel. ^{35}S-Labeled proteins were detected using O-XAR films (Eastman Kodak, MA) exposed for 1 to 10 days at -70 °C.

RNase Protection Analysis

RNA extraction was performed as described (Qiagen). The RNA probe for ICER was generated from pJL5 by XhoI or XbaI digestion which corresponds to full-length cDNA of human ICERII described previously (167). RNA probes hAPO3 and mAPO3 were purchased from PharMingen (San Diego, CA) and labeled with [α-^{32}P] UTP using reagents from an RNA probe kit (Ambion). These probes were used for RNase protection studies according to the protocol provided by Ambion (RPAII Ribonuclease Protection Assay kit).

Expression and purification of recombinant proteins

Human ICERII cDNA was subcloned into the pGEXKG vector (Pharmacia) and expressed in bacteria as a GST-fusion protein. Purification of ICER was carried out with minor modifications according to the protocol previously established for CREB (187). NFATpXS(1-187) encompassing the minimal DNA-biding domain of NFATp (gift from Dr. A. Rao) was expressed in bacteria as a hexahistidine-tagged protein and purified as reported previously (180).

Electrophoretic gel mobility shift assay

Binding reactions were performed in a 15 µl reaction volume containing 20 mM HEPES, 1 mM $MgCl_2$, 50 mM KCl, 12% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.2 µg poly (dI-dC) as an non-specific competitor, and

recombinant proteins as indicated. ^{32}P-labeled oligonucleotides were added and incubated for 10 min at room temperature. Samples were run on a 4% polyacrylamide gel in 0.5 x TBE at 200 V for 2 h following a 2 h pre-run at 4°C. The dried gels were exposed for autoradiography overnight. The oligonucleotides encompassing NFAT composite sites of the following human promoters were used:

SEQ ID NO. 15: FasL prox 5'-gatccTAGCTATGGAAACTCTATAa-3'

SEQ ID NO. 16: FasL dist 5'-gatccCTGGGCGGAAACTTCCAGGa-3'

SEQ ID NO. 17. IL-2 (-160) 5'-ctagaAAAGAATTCCAAAGAGTCATCAGAAa-3';

SEQ ID NO. 37 GM-CSF (-420) 5'-gatccCATCTTTCTCATGGAAAGATGACATCAGGGAa-3';

Lower case letters indicate overhangs for SpeI/XbaI recognition sites (IL-2) or BamHI/BglII recognition sites (FasL, GM-CSF).

Transient Overexpression in 2B4 T cell hybridoma

Transfection assays were performed by the modified DEAE-dextran technique in 96 well CulturPlates (Packard, NJ) coated or not with antiCD3 monoclonal antibody 2C11. Typically, 2 x 10^6 cells transfected with 2 μg of the reporter and the same amount of ICER expression vector were distributed into two wells precoated with 2C11 and two uncoated wells. After 12 to 16 hrs LucLite assay (Packard, NJ) was used for evaluation of luciferase activity and luminiscence was quantified on TopCount (Packard, NJ). Chloramphenicol acetyltransferase assays and quantification methods are described elsewhere (188).

Example 23

ICER Induction Observed Early After Forskolin Treatment in Freshly Isolated Human Peripheral Blood T Lymphocytes is Limited in B Lymphocytes

First we sought to characterize distinct populations amongst human peripheral blood lymphocytes (PBLs) with respect to whether lymphocyte subpopulations show differential regulation of ICER as might be expected if ICER plays a functionally important role in lymphocytes. Immunoprecipitations using CREM-specific antiserum with respect to the CD3$^+$ and CD19$^+$ lymphocyte subsets indicate that only the lymphocytes enriched for CD3$^+$ T cells show elevated background levels of ICER protein accompanied by significant early induction of ICER after three hours of forskolin treatment (Fig. 18). Importantly, no other CREM isoforms could be detected (see analysis of Jurkat T cells below in Fig. 18 and Fig. 23B) in agreement with the absence of CREM mRNA based on data from RNase protection studies reported previously (167). Forskolin-mediated ICER induction in the peripheral blood lymphocytes prior to separation (Fig. 1, lane 1 to 4) was significantly increased in the cell population enriched for CD3$^+$ T cells (Fig. 1, lanes 5 to 8) while negligible in the CD3 pass-through population (Fig. 1, lanes 9 to 12) suggesting that CD3$^+$ T lymphocytes could be the population responsible for inducible forskolin-mediated ICER expression detectable in freshly prepared unseparated human peripheral blood lymphocytes after three hours of treatment. Moreover, the lymphocyte population enriched for CD19$^+$ B cells did not show significantly increased ICER levels in comparison with unseparated PBLs and was refractory to forskolin treatment (Fig. 18, lanes 13 to 16). In contrast to fresh peripheral blood lymphocytes, ICER was not detectable in the control leukemic Jurkat T cell line prior to forskolin treatment (Fig. 18,

lanes 21 and 22) in agreement with RNase protection data (see Fig. 23B, lane 1) and only limited levels of ICER mRNA (Fig. 23B, lane 2) and protein could be detected after forskolin treatment in Jurkat T cells (Fig 18, lanes 23 and 24) indicating that signal detected in immunoprecipitations is ICER specific. The differential regulation of ICER expression found in these studies supports the notion that ICER might act as a potentially important molecule in T lymphocyte function.

Example 24

Forskolin- mediated Transcriptional Attenuation of FasL Expression in Activated 2B4 T Cell Hybridoma as well as Human Peripheral Blood T Lymphocytes Correlates with ICER Induction

The preceeding experiments indicated that ICER is efficiently expressed in T cells. In order to study the role of ICER expression in cells of T cell lineage further we utilized the 2B4 T cell hybridoma. It was reported previously that forskolin can effectively counteract apoptosis in phorbol ester-ionomycin treated 2B4 hybridoma T cells (which in many respects mimics TcR-mediated activation triggering AICD due to potent up-regulation of FasL expression) by transcriptional attenuation of FasL expression (161, 162). To elucidate the mechanism of forskolin-mediated inhibition of FasL expression, we examined whether forskolin can induce ICER in phorbol ester-ionomycin activated 2B4 T cells (Fig 19A). In the presence of forskolin, phorbol ester-ionomycin treatment even further potentiated induction of ICER mRNA in activated 2B4 T cells (Fig. 19A, lanes 1 to 6) which in the presence of ICER failed to express FasL message (Fig. 19A and B, lanes 1 to 6). In contrast, in the absence of forskolin, high levels of FasL mRNA were accompanied with little or no ICER-specific expression (Fig 19A and B, lanes 7 to 12). Time course data in activated 2B4 T cell hybridoma indicate a tight correlation between down-regulation of FasL expression and induction of the potent transcriptional repressor ICER consistent with the possibility that forskolin-mediated ICER expression could cause transcriptional attenuation of FasL expression.

In order to assess relevance of our observations in mouse 2B4 T cell hybridoma to fresh human T cells, we isolated human peripheral blood T lymphocytes using a depletion strategy rather than positive selection to further separate $CD3^+$ cells by elimination of the double positive $CD56^+$ $CD3^+$ lymphocyte population expressing NK cell marker(s). The background levels of ICER expression seen prior to forskolin treatment in $CD3^+$ selected T lymphocytes containing $CD56^+$ $CD3^+$ lymphocyte population raised the possibility that non-T cells such as NK lymphocytes expressing $CD56^+$ could also express ICER (Fig. 18). Indeed, negatively selected T cells depleted of $CD56^+$ NK cells showed very low levels of ICER mRNA prior to forskolin treatment (Fig. 20B, lane 4) while phorbol ester-ionomycin treatment alone yielded an increase in ICER mRNA in these cells (Fig. 20, lane 2). This could be possibly due to contaminating cells since there is no significant ICER expression detectable in the absence of contaminating cells in the 2B4 T cell line (Fig. 19A, lanes 7 to 12) nor the Jurkat T cell line (data not shown). In the presence of forskolin, low levels of FasL message correlated with increased levels of ICER message in phorbol ester-ionomycin activated T cells (Fig. 20, lane 1) while in phorbol ester-ionomycin activated T cell population without forskolin increased FasL expression correlated with decreased ICER mRNA (Fig 20, lane 2).

Example 25

Induction of FasL Expression in Phorbol Ester Activated Human Peripheral Blood NK Lymphocytes is Accompanied by Cessation of ICER Expression

To examine ICER expression in cells of NK lineage, human lymphocyte populations enriched for CD56$^+$ cells were evaluated. Interestingly, they were found to exhibit elevated levels of ICER mRNA prior to forskolin treatment with dramatic decrease of ICER mRNA after three hours of phorbol ester treatment which is coincident with induction of FasL, FAP (PNP1, protein tyrosine phosphatase 1E), and TRADD (TNF receptor-1 associated death domain protein) expression (Fig. 21). Levels of individual mRNAs were assessed by RNase protection assay used for evaluation of messages of multiple constituents of Fas and TNF pathways in human lymphoid cells (FLICE, FasL, Fas, FADD, FAP, FAF, Fas2L, TNFRp55, TRADD, and RIP) (Fig. 21A). FasL message was dramatically increased after phorbol ester treatment while levels of ICER mRNA were decreased to background (Fig. 21B).

To test whether elevated levels of ICER message observed in freshly isolated human peripheral blood lymphocytes enriched for CD56$^+$ NK cells correlate with elevated levels of ICER protein in human peripheral blood NK lymphocytes prior to forskolin treatment, we performed immunoprecipitations using CREM-specific antiserum (Fig. 22A). Data confirmed that ICER mRNA in freshly isolated NK lymphocytes is efficiently translated into ICER protein prior to forskolin treatment. Moreover, comparison of ICER mRNA in two human NK cell lines NK3.3 (185) and NK92 (186) with the Jurkat leukemic T cell line, which served as a control human T cell line, indicated that both NK3.3, and to lower extent also NK92, showed elevated levels of ICER mRNA prior to forskolin treatment in RNase protection assay (Fig. 22B, lanes 5 and 3). As T cell controls, neither the Jurkat T cell line (Fig. 22B, lanes 1 and 2) nor a panel of other human T cell lines tested previously (MOLT3, MOLT4, HUT78, and CEM) (167) expressed significant levels of ICER mRNA prior to forskolin treatment. Together, these experiments showed a distinct pattern of ICER expression in CD56$^+$ NK cells as opposed to CD3$^+$T cells or CD19$^+$ B cells and further supported the hypothesis that ICER may regulate FasL expression by the reciprocal balance of the expression of the two.

Example 26

Proximal NFAT Binding Site of the Fas Ligand Promoter Binds ICER Either Alone or in Complex with NFAT DBD

To further investigate the possible role of ICER in Fas ligand gene expression, we examined the binding of bacterially expressed ICER to two NFAT motifs of the Fas ligand promoter reported to be essential for the full induction of the Fas ligand gene (Fig. 23A) (173, 174). The binding specificity of recombinant ICER was evaluated using a CREM-specific antiserum (CS4) (168) that "supershifts" ICER bound to specific oligonucleotides containing individually proximal and distal NFAT motifs of Fas ligand promoter and two control NFAT/AP-1 motifs in the IL-2 promoter in position (-160) and GM-CSF promoter in position (-420) (Fig. 23A and B). Both of these control motifs can bind ICER either directly via AP-1 like sequence adjacent to NFAT motif and/or indirectly via interaction of ICER with NFAT DBD (168), which is both necessary and sufficient for NFAT DNA binding as well as complex formation with ICER (168) or AP-1 (181). ICER and ICER containing complexes can be competed by unlabeled oligonucleotides containing CRE motifs (168). Similarly to the case in proximal NFAT motif of the IL-2 promoter, we observed specific direct binding of ICER to the proximal NFAT motif of the FasL promoter which in the presence of NFAT DBD yielded a

ternary NFAT/ICER complex (Fig. 23C, lanes 1 to 3). Unlike the proximal NFAT motif in Fas ligand promoter, the distal NFAT motif fails to support significant level of ICER binding and/or formation of NFAT/ICER ternary complex (Fig. 23C, lanes 4 to 6). Instead, the distal NFAT motif supports, at least in vitro, high affinity NFAT DBD binding accompanied by NFAT DBD dimer formation which is consistent with the proposed dominant role of distal NFAT motif in activation of Fas ligand expression (15, 16). These experiments provide further evidence supporting ICER as a regulatory molecule in FasL expression.

Example 27

Ectopically Expressed ICER Represses Transcription from Activated Human FasL Luciferase Reporter

To determine whether ICER expression can mediate the effect of forskolin in transcriptional attenuation of FasL promoter observed in 2B4 T cell hybridoma and peripheral blood T and NK lymphocytes, different ICER isoforms (ICERII, ICERIIγ, ICERI, and ICERIγ) were ectopically expressed in 2B4 hybridoma T cells in transient transfection assays. Expression of ICER down-regulated both human FasL promoters with or without distal NFAT motif (Fig. 24A) activated by a monoclonal antibody 2C11 (with specificity for the CD3ε subunit of the CD3 complex) (Fig. 24B). Ectopic expression of either isoform of ICER had no significant effect on VP16-mediated transactivation of (3xGAL4)-CR-CAT (168) (data not shown). Moreover, FasL reporter containing the proximal NFAT motif alone was found even more susceptible to ICER-mediated repression in accordance with the higher affinity of proximal NFAT motif for ICER binding and complex formation documented in gel shift assays (Fig. 23, and Fig. 23B). Thus, ICER can be induced by, and substituted for, forskolin in the transcriptional down-regulation of FasL reporter induced by antiCD3ε stimulation in 2B4 T cells.

The mechanism of cAMP-mediated inhibition of FasL expression in activated T lymphocytes is correlated with cAMP-mediated induction of the powerful transcriptional repressor - ICER.

Footprinting and electrophoretic mobility shift analyses revealed two NFAT sites within FasL promoter, proximal (from -126 to -144), and distal (from -263 to -283) as essential for high levels of FasL expression in T lymphocytes (173, 174). The role of NFAT in the activation of FasL promoter was further strengthened by the observations that activated T lymphocytes from NFATp knock out mice (175) failed to express FasL in T lymphocytes and showed typical signs of splenomegaly inherent to *gld* mice models (165) suggesting that in T cell environment NFAT plays an important role in activation of FasL expression. Our observations indicate that similar to the proximal NFAT motif in the IL-2 promoter (168), the proximal NFAT site in the FasL promoter has the capacity to associate with NFAT DBD (181) and ICER to form inhibitory NFAT/ICER complex. Moreover, a FasL reporter containing the proximal NFAT motif alone is highly susceptible to ICER-mediated repression in transient transfection assays in agreement with the ability of ICER to bind the proximal NFAT motif either alone or in the complex with NFAT DBD. To prove that ICER expression rather than high intracellular levels of cAMP elicited by forskolin could be responsible for observed inhibitory effect on FasL promoter, ICER and FasL reporter were co-expressed in 2B4 T cells activated by antiCD3ε antibody. These transient transfections indicate that significant down-regulation of activated FasL promoter in 2B4 T cell hybridoma can be observed based on expression of different isoforms of ICER (ICERII, ICERIIγ,

ICERI, and ICERIγ) and/or FasL reporters. Thus, even in the absence of high intracellular levels of cAMP, the transcriptional repressor ICER can be sufficient for the transcriptional attenuation of FasL promoter activated by antiCD3ε stimulation in 2B4 T cell hybridoma.

We have previously identified human medullary thymocytes as a forskolin-responsive population which, in contrast to the less mature cortical thymocytes, is capable of cAMP-mediated ICER induction *ex vivo* (167, 168). Here we demonstrate that the ability of forskolin to mediate early ICER induction is retained also in freshly isolated subpopulations of human peripheral blood lymphocytes enriched for $CD3^+$ mature T lymphocytes while present in modest fashion in $CD19^+$ selected B lymphocytes or $CD56^+$ NK lymphocytes. Despite the observation that neither NK nor B lymphocytes show significant forskolin-mediated ICER induction, they differ dramatically in their pre-existing levels of ICER prior to forskolin treatment. It seems that mostly T lymphocytes constitute the forskolin-responsive ICER inducible population of lymphocytes although background levels of ICER in positively selected $CD3^+$ T lymphocytes are usually higher in comparison to T lymphocytes isolated by depletion strategy. Aside from CD3 triggered ICER induction another possible explanation of this observation is related to the population of large granular lymphocytes expressing both T ($CD3^+$) and NK ($CD56^+$) cell markers on their surface (189) which could be retained amongst $CD3^+$ positively selected T cells, contributing to elevated background levels of ICER. These double positive cells which are depleted during negative isolation of T cells show usually elevated background levels of ICER mRNA. Nevertheless, both positively and negatively selected T cell populations contain low numbers of contaminating cells which after phorbol ester and ionomycin treatment may contribute indirectly to the elevated levels of ICER (e.g. by release of cAMP agonists such as prostaglandins). In contrast, phorbol ester-ionomycin treatment in T cell lines such as 2B4 T cell hybridoma (Fig. 19A) or Jurkat T cell line (data not shown) does not yield ICER mRNA and these T cell lines require forskolin treatment for ICER induction.

Unlike B cells or medullary thymocytes (167), freshly prepared human $CD56^+$ NK cells show significant expression of ICER prior to forskolin treatment which could be responsible at least in part for ICER-specific signal detectable in elutriated peripheral blood lymphocytes observed before subsequent separations both on mRNA and protein levels. Importantly, the human NK cell lines NK 3.3 and to lower extent also NK 92 which like freshly isolated human NK cells express CD56 on their surface (185, 186), exhibit increased levels of ICER mRNA prior to the forskolin treatment although they have not been exposed to CD56-specific antibodies used for isolation of fresh human NK cells.

The engagement of stimuli involved in Fas ligand-Fas pathway in human NK cells have been mimicked for the purpose of this study by phorbol ester treatment leading to profound increase of Fas ligand expression accompanied by reported increased ability of NK cells to kill in cytotoxic assays (190). While phorbol ester-mediated Fas ligand expression is likely to be independent of NFAT-driven expression, cessation of ICER expression correlates with de-repression of Fas ligand expression. Although, we cannot exclude the possibility that other factors such as positive selection for $CD56^+$ NK cells may contribute to the ICER-specific signal, cessation of ICER expression and subsequent up-regulation of FasL expression is consistent with postulated repressive role of ICER in NK cells even in the absence

of NFAT function. Thus, it is conceivable that ICER acts as a transcriptional repressor of Fas ligand expression which could be effective under the circumstances when transcriptional factors other than NFAT are responsible for Fas ligand expression. It is also possible that ICER may participate in transcriptional modulation of other yet unidentified transcription factors acting in trans on FasL promoter.

The role of ICER in cAMP-mediated transcriptional attenuation of FasL promoter seems to be strikingly similar to the role of recently cloned Glucocorticoid Inducible Leucine Zipper (GILZ) implicated in dexamethasone-mediated transcriptional attenuation of FasL (191). This transcriptional repressor showing remarkable homology with leucine zipper of ICER is induced in lymphoid tissues by dexamethasone. Moreover, induction of Glucocorticoid Inducible Leucine Zipper - GILZ correlates with dexamethasone-induced transcriptional attenuation of FasL expression which could lead to inhibition of AICD (191) in analogous fashion as ICER induction could lead to forskolin-mediated inhibition of AICD (161). Thus, signaling through diverse agonists such as cAMP or dexamethasone leading ultimately to transcriptional attenuation of FasL promoter could be conferred in T lymphocytes by induction of at least two independent transcriptional repressors - ICER and GILZ.

V. Use of Agents which Modulate ICER Activity as Therapeutic Agents or Reagents for Studying Immune Cell Activity

The above results demonstrate that ICER is involved in regulating immune cell activity. As discussed above, ICER-mediated inhibition of immune cell activity may be involved in the progression of a variety of clinical conditions. In particular, ICER-mediated inhibition of immune cell activity may contribute to the progression of conditions such as cancer or diseases resulting from infection with pathogenic organisms.

Accordingly, agents which reduce the level of ICER-mediated inhibtion of immune cell activity represent attractive therapeutics for treating conditions which result from or are exacerbated by the inhibition of immune cell activity.

In addition, agents which increase the level of ICER activity represent attractive therapeutics for treating conditions conditions which result from or are exacerbated by the over-activity of immune cells.

Furthermore, agents which reduce or increase the level of ICER-mediated inhibition of immune cell activity are valuable reagents for studying and characterizing the molecular mechanisms which govern the activity of immune cells and the progression of diseases resulting from or exacerbated by the inhibition of immune cell activity using in vitro or in vivo model systems.

As discussed above, tumors have been shown to secrete factors such as IL-10, PGE2, and others which reduce immune cell activity (17,18), or to induce host cells to secrete such factors (19-21). In addition to the experiments described above, which demonstrate ICER's involvement in the inhibition of immune cell activity, ICER's involvement in the tumor-mediated inhibition of immune cell activity may be further characterized as described below. In particular, the following procedures may be used to characterize ICER's involvement in tumor-mediated inhibition of immune cells such as T lymphocytes, B lymphocytes, NK cells, monocytes, dendritic cells, and antigen presenting cells.

Example 28

Characterization of ICER's Involvement in Tumor-mediated Inhibition of Immune Cell Activity

Substances obtained from or secreted by viable tumor cells, or obtained from killed tumor cells, tumor cell lysates, or supernatants from tumor cells are incubated with normal immune system cells such as T lymphocytes, B lymphocytes, NK cells, monocytes, dendritic cells, and antigen presenting cells. The immune activities of the cells are measured using standard techniques such as cytokine assays (to measure secreted cytokines), proliferation assays, stimulation assays, and target lysis assays.

The expression of ICER mRNA and protein is measured in the cells suppressed by tumors above to determine whether sustained ICER expression occurs during such inhibition. In particular, ICER expression is measured in cells suppressed by tumors under conditions in which PGE2 is secreted or induced by tumor cells. A variety of techniques, such as blocking antibodies, inhibitors of PGE2 receptors, etc. are use to determine which particular factors are responsible for such inhibition and sustained ICER expression.

The above experiments may be performed in vitro or in in vivo models to characterize the mechanisms and factors involved in immune cell activity.

ICER's involvement in the inhibition of immune cell activity caused by infectious organisms may also be characterized using the procedures described above. As discussed above, infectious pathogens pathogens are known to produce factors such as viral IL-10 or cAMP-stimulating factors (22,23) which inhibit immune cell acitivity.

In such procedures, substances obtained from or secreted by cells infected with a pathogenic organism, or obtained from infected cells, infected cell lysates, or supernatants from infected cells are incubated with normal immune system cells such as T lymphocytes, B lymphocytes, NK cells, monocytes, dendritic cells, and antigen presenting cells. The immune activities of the cells are measured using standard techniques such as cytokine assays (to measure secreted cytokines), proliferation assays, stimulation assays, and target lysis assays.

The expression of ICER mRNA and protein is measured in the immune cells whose activity is suppressed by through the action of an infectious organism to determine whether sustained ICER expression occurs during such inhibition. A variety of techniques, such as blocking antibodies, inhibitors of cytokine receptors, and other techniques familiar to those skilled in the art are use to determine which particular factors are responsible for such inhibition and sustained ICER expression.

The above experiments may be performed in vitro or in in vivo models to characterize the mechanisms and factors involved in immune cell activity.

As discussed above, agents which reduce or increase the level of ICER activity in immune cells may be used to characterize the mechanisms and factors involved in controlling immune cell activity using in vitro or in vivo model systems as well as to treat conditions characterized by or exacerbated by inhibition or stimulation of immune cell activity.

A variety of agents may be used to reduce or increase the level of ICER activity in immune cells such as T lymphocytes, B lymphocytes, NK cells, monocytes, dendritic cells, and antigen presenting cells. These agents include

ribozymes, antisense nucleic acids, triple helix forming nucleic acids, and other factors which increase or decrease transcription from the ICER promoter.

Ribozymes are RNA molecules which possess an endonuclease activity. The ribozymes for use in reducing ICER activity levels contain one or more catalytic regions capable of cleaving one or more target sites in mRNAs encoding one or more ICER isoforms. In addition, to provide specificity for ICER mRNA, the ribozymes include one or more targetting regions which are complementary to one or more sequences in the ICER mRNA.

Any of the types of ribozymes familiar to those skilled in the art may be used to reduce ICER activity levels. These include "hammerhead" ribozymes comprising a caatalytic core of nine bases, a double stranded step-loop structure, and two regions on either side of the catalytic core which are complementary to the target mRNA. (Haseloff and Gerlach (1988) Nature 334:585-591). Alternatively, ribozymes such as those described in U.S. Patent No. 5,116,742 may be used.

In some embodiments, the ribozymes for reducing ICER activity levels may have multiple catalytic regions, such as those described in U.S. Patent No. 5,635,385. In further embodiments, the ribozymes may include one or more rigid linkers, such as those described in U.S. Patent Nos. 5,679,555 and 5,650,502 to increase their stability in vivo. In other embodiments, the ribozymes may include one or more 2'-O-alkylated nucleotides such as those described in U.S. Patent No. 5,545,729 to enhance their stability in vivo.

The ability of different ribozymes to reduce ICER activity levels is evaluated in a variety of types of immune cells, including T lymphocytes, B lymphocytes, NK cells, monocytes, dendritic cells, and antigen presenting cells, as described below. Example 29 describes the analysis of the ability of different ribozymes to inhibit T cell acitivity.

Example 29

Analysis of the Ability of Different Ribozymes to Inhibit T cell Acitivity

As discussed above, normal human T cells (such as freshly isolated peripheral blood $CD4^+$ T cells) express ICER mRNA and ICER protein after exposure to the cAMP agonists forskolin and sustained ICER mRNA expression occurs after combined treatment with ionomycin plus forskolin. Treatment of such T cells with cAMP-stimulating agents or agonists such as forskolin or PGE2 also results in marked prolonged blockade of T cell proliferation whether the proliferation is stimulated by calcium ionophore or by specific antigenic restimulation. Accordingly, the ability of ribozymes to reduce ICER-mediated inhibition of T cell activity may be measured by determining their ability to block the inhibition of T cells treated with forskolin, PGE2 or ionomycin plus forskolin.

A set of ribozymes having different catalytic sequences capable of recognizing different cleavage sites and/or different targetting sequences capable of directing the ribozyme to different sites ICER mRNAs encoding one or more ICER isoforms is designed using conventional techniques. As a control, ribozymes incapable of cleaving ICER mRNA, such as ribozymes having targetting sequences identical to the sequence of one or more forms of ICER mRNA such that they do not form a duplex with ICER mRNA may be used. Alternatively, the control ribozyme may be a ribozyme which targets an mRNA other than an ICER mRNA.

The ribozymes are then prepared using any of the techniques familiar to those skilled in the art. In particular, the ribozymes may be synthesized conventional chemical synthesis procedures. Alternatively, the ribozymes may be prepared by performing an in vitro transcription reaction on a linearized vector comprising a nucleotide sequence encoding the ribozyme operably linked to a promoter. For example, the promoter directing the in vitro transcription reaction may be the T7 promoter or the SP6 promoter.

Each of the ribozymes are then introduced into the T cells at a range of concentrations. The ribozymes may be introduced using any of the techniques familiar to those skilled in the art. For example, the ribozymes may be introduced into T cells as naked molecules. Alternatively, the ribozymes may be introduced into the T cells using lipofection.

The effect of each ribozyme on ICER activity in T cells may be evaluated using a variety of systems. In one assay, the effect of each ribozyme on ICER activity in T cells is determined by quantitating its ability to reduce or disrupt the forskolin-induced inhibition of T cell proliferation. In such assays $CD4^+$ T cells are prepulsed with the ribozymes to be screened, or with combinations of ribozymes prior to exposure to cAMP-stimulating agents or agonists such as forskolin. Immunoprecipitation and/or RNAse protection assays are performed to identify those ribozymes which are capable of potently blocking forskolin-induced ICER expression in these T cells. The results obtained with each of the ribozymes being screened are compared to those in cells which received control ribozymes. Ribozymes which reduce ICER expression levels or mRNA levels to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces ICER expression to the greatest extent is used as such a therapeutic or reagent.

Based on ICER's ability to inhibit the IL-2 gene's enhancer/promoter function (5) , as well as cyclic AMP agonists' diverse impairment of other T cell functions (27), ICER ribozymes may also be used to reduce or prevent ICER/cAMP-mediated inhibition of T cell cytokine secretion as well as cAMP-mediated inhibition of T cell signal transduction. In yet another approach, the effect of each ribozyme on ICER activity in T cells is determined by quantitating its ability to reduce or disrupt cAMP-induced suppression of T cell function using standard assays for measuring T cell proliferation, cytokine release asays, signal transduction and target lysis assays and comparing the results to those obtained with control ribozymes. Those ribozymes which reduce ICER expression levels or mRNA levels to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces ICER expression to the greatest extent is used as such a therapeutic or reagent.

Alternatively, vectors comprising nucleotide sequences encoding each of the test ribozymes operably linked to a promoter are introduced into T cells, such as freshly cultured $CD4^+$ anti-TETANUS T cells. The vector may comprise any of the vectors conventionally used to express nucleic acids in immune cells, including retroviral vectors (28,29), episomal vectors, vectors which are stably integrated into the genome of the host cell, or vectors which are transiently present in the host cell. As a control, a vector encoding a ribozyme having targetting sequences identical to the sequence of one or more forms of ICER mRNA such that it does not form a duplex with ICER mRNA or a

ribozyme which targets an mRNA other than an ICER mRNA is introduced in the T cells. Immunoprecipitation with ICER antibodies or RNAse protection assays is performed to measure the levels of ICER protein or intact ICER mRNA present in T cells containing vectors encoding each of the test ribozymes. The results are compared to the levels in T cells receiving control ribozymes. Ribozymes which reduce ICER expression levels or mRNA levels to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces ICER expression to the greatest extent is used as such a therapeutic or reagent.

In another approach, the ability of the ribozymes to inhibit T cell activity in vivo is determined as follows. Test and control ribozymes are introduced into T cells using any of the above methods (i.e. naked nucleic acids, lipofection, and stable or transient expression vectors). The T cells are then administered to same-strain tumor bearing mice to measure the ability of the ribozymes to inhibit ICER-mediated inhibition of the immune response. The results obtained with each of the test ribozymes are compared to those obtained with the controls. Ribozymes which reduce ICER-mediated inhibition of the immune response to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces ICER-mediated inhibition of the immune response to the greatest extent is used as such a therapeutic or reagent.

Ribozymes may also be used to reduce ICER-mediated inhibition of immune cell activity in immune cells other than T cells, such as B cells, NK cells, dendritic cells, or antigen presenting cells. Methods for identifying ribozymes which effectively reduce ICER-mediated inhibition of antigen presenting cell activity are described below.

Example 30

Methods for Identifying Ribozymes which effectively reduce ICER-mediated inhibition of Antigen Presenting Cell Activity

A variety of ICER ribozymes are screened for the ability to reduce or prevent ICER-mediated inhibition of antigen presenting cells, such as monocytes or dendritic cells using the procedures described above for T cells. cAMP-stimulating agents or agonists such as PGE2 and forskolin have been shown to have a variety of inhibitory effects on antigen-presenting cells (APC), including inhibition of IL-12 secretion and inhibited upregulation of the costimulatory molecule B7.1 (see above). APC, such as monocytes and dendritic cells (DC), are stimulated with cAMP stimulating agents and treated with the ICER ribozymes or control ribozymes as described in the T cell assays above.

In one approach, ICER-mediated inhibition may be measured by measuring the PGE2/forskolin-induced inhibition of calcium-ionophore-stimulated CD80 (B7.1) expression in peripheral blood human monocytes (4). Ribozymes which reduce the inhibition of antigen presenting cell activity to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces the inhibition of antigen presenting cell activity to the greatest extent is used as such a therapeutic or reagent.

The ability of ICER ribozymes to reduce or prevent the ICER-mediated inhibition of B lymphocyte activity may also be confirmed as follows.

Example 31

Evaluation of the Ability of ICER Various ICER Ribozymes to Reduce or Prevent ICER-mediated Inhibition of the Activity of B Lymphocytes

Experiments similar to those described above for T cells and APCs are performed with B lymphocytes. Inducing cAMP in B lymphocytes by diverse stimuli has been demonstrated by others to have a variety of inhibitory effects, including induction of B cell unresponsiveness during a synchronous antigenic stimulus (see above). ICER ribozymes and control ribozymes are transiently or introduced into B lymphocytes treated with cAMP agonists as described above.

Ribozymes which reduce the inhibition of B cell activity to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces the inhibition of B cell activity to the greatest extent is used as such a therapeutic or reagent.

The ability of ICER ribozymes to reduce or prevent the ICER-mediated inhibition of NK cell activity may also be confirmed as follows.

Example 32

Evaluation of the Ability of ICER Various ICER Ribozymes to Reduce or Prevent ICER-mediated Inhibition of the Activity of NK Cells

Inducing cAMP in cultured NK lymphocytes inhibits NK target-directed lysis (see above). ICER ribozymes and control ribozymes are transiently or stably introduced into NK cells treated with cAMP agonists as described above. The extent of inhibition of target-directed lysis by NK cells by cAMP agonists is determined in NK cells receiving the ICER ribozymes and control ribozymes. Ribozymes which reduce the inhibition NK cell activity to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the ribozyme which reduces the inhibition of NK cell activity to the greatest extent is used as such a therapeutic or reagent.

Antisense nucleic acids may also be used to reduce the level of ICER activity in immune cells. ICER antisense to various ICER isoforms has been stably transfected into several cell lines (18,24,25). It has been reported that select cell lines can be stably transfected with a construct which results in perpetual intracellular overexpression of ICER antisense, thereby blocking forskolin-inducible ICER synthesis in the transfected cells (18). Such cell lines do not experience lethality or apparent toxicity from stable ICER-antisense expression *per se*, but ICER-dependent effects of cAMP-stimulating agents or agonists are blocked. Accordingly, normal human T cells can be stably transfected with vectors hyperexpressing ICER antisense to reduce or prevent the inhibitory effects of ICER induced by tumors and other pathogens. Because certain constructs block endogenous ICER induction in some cell lines (25) but not in others

(18), a variety of antisense sequence(s) are screened to identify those that are efficient for reducing or preventing endogenous induction, and to determine whether each ICER isoform has different suppressibility by a particular antisense sequence.

ICER antisense nucleic acids which reduce cAMP-induced inhibition of the activity of immune cells, T lymphocytes, B lymphocytes, NK cells, monocytes, dendritic cells, and antigen presenting cells, are identified as follows.

Example 33

Identification of ICER Antisense Molecules which Reduce

ICER-mediated Inhibition of T Cell Activity

As discussed above, normal human T cells (such as freshly isolated peripheral blood $CD4^+$ T cells) express ICER mRNA and ICER protein after exposure to the cAMP agonists forskolin and sustained ICER mRNA expression occurs after combined treatment with ionomycin plus forskolin. Treatment of such T cells with cAMP-stimulating agents or agonists such as forskolin or PGE2 also results in marked prolonged blockade of T cell proliferation whether the proliferation is stimulated by calcium ionophore or by specific antigenic restimulation. Accordingly, the ability of antisense nucleic acids to reduce ICER-mediated inhibition of T cell activity may be measured by determining their ability to block the inhibition of T cells treated with forskolin, PGE2 or ionomycin plus forskolin.

A set of nucleic acids complementary to all or a portion of one or more forms of ICER mRNA is prepared. In some embodiments, the antisense nucleic acids are oligonucleotides comprising less than 50 consecutive nucleotides complementary to one or more forms of ICER mRNA. In other embodiments the antisense nucleic acids comprise at least 75 consecutive nucleotides complementary to one or more forms of ICER mRNA. In further embodiments, the antisense nucleic acids comprise at least 100 consecutive nucleotides complementary to one or more forms of ICER mRNA. In other embodiments, the antisense nucleic acids comprise at least 150 consecutive nucleotides complementary to one or more forms of ICER mRNA. In further embodiments, the antisense nucleic acids comprise at least 200 consecutive nucleotides complementary to one or more forms of ICER mRNA. In still further embodiments, the antisense nucleic acids comprise more than 200 consecutive nucleotides complementary to one or more forms of ICER mRNA. In additional embodiments, the antisense nucleic acids comprise nucleic acids which are complementary to the total sequence of one or more forms of ICER mRNA.

In some embodiments, the antisense nucleic acids may comprise oligonucleotide sequences which constitute "antisense" to various portions of the ICER gene, including the initiation codon common to all four isoforms of ICER, the termination codon present in individual isoforms, or any other sequence present in one or more ICER isoforms. Such oligonucleotides may be synthesized using conventional techniques. In other embodiments, an antisense sequence common to all isoforms of ICER which runs from the region approximately 60 oligonucleotides upstream to the initiation codon, continuing through the first translated 24 oligonucleotides in the ICER specific domain (-60 through +24 on the ICER sequence published in Figure 2 of Fujimoto et al. (26) is used.

Where the antisense sequence is longer than 50 bases in length, it may be synthesized by performing in vitro trancription reactions on vectors in which a nucleic acid encoding the antisense sequence is operably linked to a promoter. Alternatively, vectors encoding the antisense nucleic acid may be transiently or stably introduced into the T cells as described above.

The effect of each antisense nucleic acid on ICER activity in T cells may be evaluated using a variety of systems. In one assay, the effect of each antisense nucleic acid on ICER activity in T cells is determined by quantitating its ability to reduce or disrupt the forskolin-induced inhibition of T cell proliferation. In such assays $CD4^+$ T cells are prepulsed with the antisense nucleic acids to be screened, or with combinations of antisense nucleic acids prior to exposure to cAMP-stimulating agents or agonists such as forskolin. Immunoprecipitation and/or RNAse protection assays are performed to identify those antisense oligonucleotide sequences which are capable of potently blocking forskolin-induced ICER expression in these T cells. The results obtained with each of the antisense nucleic aicds being screened are compared to those in cells which received control antisense nucleic acids. Antisense nucleic acids which reduce ICER expression levels or mRNA levels to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces ICER expression to the greatest extent is used as such a therapeutic or reagent.

Based on ICER's ability to inhibit the IL-2 gene's enhancer/promoter function (5) , as well as cyclic AMP agonists' diverse impairment of other T cell functions (27), ICER antisense may also be used to reduce or prevent ICER/cAMP-mediated inhibition of T cell cytokine secretion as well as cAMP-mediated inhibition of T cell signal transduction. In yet another approach, the effect of each antisense nucleic acid on ICER activity in T cells is determined by quantitating its ability to reduce or disrupt cAMP-induced suppression of T cell function using standard assays for measuring T cell proliferation, cytokine release asays, signal transduction and target lysis assays and comparing the results to those obtained with control antisense nucleic acids. Those antisense nucleic acids which reduce ICER expression levels or mRNA levels to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces ICER expression to the greatest extent is used as such a therapeutic or reagent.

Alternatively, vectors comprising nucleotide sequences encoding each of the test antisense nucleic acids operably linked to a promoter are introduced into T cells, such as freshly cultured $CD4^+$ anti-TETANUS T cells. The vector may comprise any of the vectors conventionally used to express nucleic acids in immune cells, including retroviral vectors (28,29), episomal vectors, vectors which are stably integrated into the genome of the host cell, or vectors which are transiently present in the host cell. As a control, a vector encoding a sense nucleic acid or an antisense nucleic acid which targets an mRNA other than an ICER mRNA is introduced in the T cells. Immunoprecipitation with ICER antibodies or RNAse protection assays is performed to measure the levels of ICER protein or intact ICER mRNA present in T cells containing vectors encoding each of the test antisense nucleic acids. The results are compared to the levels in T cells receiving control antisense nucleic acids. Antisense nucleic acids which reduce ICER expression levels or mRNA levels to a statiscally significant extent may be used as therapeutics or

as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces ICER expression to the greatest extent is used as such a therapeutic or reagent.

In another approach, the ability of the antisense nucleic acids to inhibit T cell activity in vivo is determined as follows. Test and control antisense nucleic acids are introduced into T cells using any of the above methods (i.e. naked nucleic acids, lipofection, and stable or transient expression vectors). The T cells are then administered to same-strain tumor bearing mice to measure the ability of the antisense nucleic acids to inhibit ICER-mediated inhibition of the immune response. The results obtained with each of the test antisense nucleic acids are compared to those obtained with the controls. Antisense nucleic acids which reduce ICER-mediated inhibition of the immune response to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces ICER-mediated inhibition of the immune response to the greatest extent is used as such a therapeutic or reagent.

Antisense nucleic acids may also be used to reduce ICER-mediated inhibition of immune cell activity in immune cells other than T cells, such as B cells, NK cells, dendritic cells, or antigen presenting cells. Methods for identifying antisense nucleic acids which effectively reduce ICER-mediated inhibition of antigen presenting cell activity are described below.

Example 34

Identification of ICER Antisense Molecules which Reduce ICER-mediated Inhibition of Antigen Presenting Cell Activity

A variety of ICER antisense nucleic acids are screened for the ability to reduce or prevent ICER-mediated inhibition of antigen presenting cells, such as monocytes or dendritic cells using the procedures described above for T cells. cAMP-stimulating agents or agonists such as PGE2 and forskolin have been shown to have a variety of inhibitory effects on antigen-presenting cells (APC), including inhibition of IL-12 secretion and inhibited upregulation of the costimulatory molecule B7.1 (see above). APC, such as monocytes and dendritic cells (DC), are stimulated with cAMP stimulating agents and treated with the ICER antisense nucleic acids or antisense nucelic acids as described in the T cell assays above.

In one approach, ICER-mediated inhibition may be measured by measuring the PGE2/forskolin-induced inhibition of calcium-ionophore-stimulated CD80 (B7.1) expression in peripheral blood human monocytes (4). Antisense nucleic acids which reduce the inhibition of antigen presenting cell activity to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces the inhibition of antigen presenting cell activity to the greatest extent is used as such a therapeutic or reagent.

The ability of ICER antisense nucleic acids to reduce or prevent the ICER-mediated inhibition of B lymphocyte activity may also be confirmed as follows.

Example 36

Evaluation of the Ability of ICER Various ICER Antisense Nucleic Acids to Reduce

or Prevent ICER-mediated Inhibition of the Activity of B Lymphocytes

Experiments similar to those described above for T cells and APCs are performed with B lymphocytes. Inducing cAMP in B lymphocytes by diverse stimuli has been demonstrated by others to have a variety of inhibitory effects, including induction of B cell unresponsiveness during a synchronous antigenic stimulus (see above). ICER antisense nucleic acids and control nucleic acids are transiently or introduced into B lymphocytes treated with cAMP agonists as described above.

Antisense nucleic acids which reduce the inhibition of B cell activity to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces the inhibition of B cell activity to the greatest extent is used as such a therapeutic or reagent.

The ability of ICER antisense nucleic acids to reduce or prevent the ICER-mediated inhibition of NK cell activity may also be confirmed as follows.

Example 37

Evaluation of the Ability of ICER Various ICER Antisense Nucleic Acids to Reduce

or Prevent ICER-mediated Inhibition of the Activity of NK Cells

Inducing cAMP in cultured NK lymphocytes inhibits NK target-directed lysis (see above). ICER antisense nucleic acids and control nucleic acids are transiently or stably introduced into NK cells treated with cAMP agonists as described above. The extent of inhibition of target-directed lysis by NK cells by cAMP agonists is determined in NK cells receiving the ICER antisense nucleic acids and control nucleic acids. Antisense nucleic acids which reduce the inhibition NK cell activity to a statiscally significant extent may be used as therapeutics or as reagents for studying the molecular mechanisms which govern immune cell activity. Preferably, the antisense nucleic acid which reduces the inhibition of NK cell activity to the greatest extent is used as such a therapeutic or reagent.

As discussed above, agents which reduce the levels of ICER activity in immune cells may be used as therapeutics to ameliorate or cure conditions which result from or which are exacerbated by ICER-mediated inhibition of immune cell activity. For example, agents which reduce the level of ICER activity in immune cells may be used to treat individuals suffering from cancer or infection with a pathogenic organism. Alternatively, agents which increase the levels of ICER activity may be used as therapeutics to ameliorate or cure conditions which result from or which are exacerbated by an elevated level of immune cell activity. Such conditions include arthritis and lupus.

In some embodiments of the above therapies, immune cells are obtained from an individual suffering from a condition to be treated. The agent which reduces or increases the level of ICER activity in immune cells is introduced into the immune cells using any of the gene therapy techniques familiar to those skilled in the art. For example, the agent may be introduced using techniques for introducing naked nucleic acids into cells, lipofection techniques, transfection techniques, or using vectors encoding the agent which are transiently or stably maintained in the host cells. The agent may be a ribozyme, an antisense nucleic acid, a composition which upregulates or downregulates the

transcription of the ICER gene, or a composition which modulates the level of ICER protein in immune cells. After the agent is introduced into the immune cells, the cells are reintroduced into the individual.

In other embodiments of the above therapies, the agent is introduced into the immune cells while the immune cells are in the individual suffering from the condition to be treated. The agent may be introduced as a naked nuceic acid, in a lipid vesicle, or via a vector encoding the agent which is stably or transiently maintained in the host cell. The agent may be a ribozyme, an antisense nucleic acid, a composition which upregulates or downregulates the transcription of the ICER gene, or a composition which modulates the level of ICER protein in immune cells.

Example 38 describes the use of an ICER ribozyme or antisense nucleic acid to reduce ICER-mediated inhibtion of T cell activity in individuals suffering from cancer, infection with a pathogenic microorganism, or from any other condition resulting from or exacerbated by ICER-mediated inhibition of T cell activity.

Example 38

Use of Antisense to Reduce or Prevent ICER-mediated Inhibition of

T Cell Activity in Subjects Suffering from Cancer

T lymphocytes are isolated from subjects suffering from cancer, infection with a pathogenic microorganism, or from any other condition resulting from or exacerbated by ICER-mediated inhibition of T cell activity. The T lymphocytes may be either $CD4^+$ T cells, $CD8^+$ T cells, or both, and may include subpopulations which possess anti-tumor specificity and function (for example, because the patient previously received a tumor vaccine to activate and increase the incidence of such subpopulations). The T cells are propagated for several days or weeks in culture, driven by a variety of stimuli such as superantigens or autologous antigen-pulsed dendritic cells (DC), and by the addition of cytokines such as rhIL-2. During this culture, the T cells are transfected with retroviral vectors containing sequences which generate ICER antisense, ICER ribozymes, or other agents which reduce ICER activity when expressed in the transduced cells. Recent advances in transfection techniques permit successful gene transfer in up to 50% of the total lymphocyte population (28,29). When the lymphocytes are propagated to adequate numbers and stable gene transfer confirmed, the lymphocytes are administered to the subjects suffering from cancer as adoptive therapy. The transduced lymphocytes are resistant to induction of sustained ICER protein synthesis in the tumor environment and thus possess markedly enhanced anti-tumor function.

To allow for elimination of the transduced lymphocytes at a later point in time, a "suicide gene" such as the Herpes Simplex-thymidine kinase (HS-tk) gene may be cotransfected with the ICER antisense or ICER ribozyme vector. The suicide gene allows HS-tk containing transduced T cells to be killed by treatment of the subject with ganciclovir (28).

Example 39 describes the use of ICER ribozymes or ICER antisense to reduce ICER-mediated inhibition of monocyte or dendritic cell activity in individuals suffering from cancer, infection with a pathogenic microorganism, or from any other condition resulting from or exacerbated by ICER-mediated inhibition of monocyte or dendritic cell activity.

Example 39

Use of Antisense to Reduce or Prevent ICER-mediated Inhibition of Monocyte and Dendritic Cell Activity in Subjects Suffering from Cancer

Peripheral blood monocytes (themselves precursors of both macrophages and dendritic cells), or cultured myeloid precursors of monocytes, macrophages and DC (such as $CD34^+$ bone marrow cells) are harvested from subjects suffering from cancer, infection with a pathogenic microorganism, or from any other condition resulting from or exacerbated by ICER-mediated inhibition of T cell activity, and briefly cultured to achieve stable transfection with retroviral vectors containing sequences which generate ICER antisense, ICER ribozymes, or other agents which reduce ICER activity when expressed in the transduced cells. To allow for elimination of the transduced myeloid cells at a later point in time, a "suicide gene" such as the Herpes Simplex-thymidine kinase (HS-tk) gene (28) may be cotransfected (see above). The cells are readministered to the patients, providing a potentially self-sustaining precursor pool of antigen-presenting cells which are resistant to induction of sustained ICER protein synthesis in the tumor environment, thereby enhancing their potential for effective antigen presentation.

The above procedures may also be performed to introduce agents which reduce ICER-mediated inhibition into other types of immune cells, including B cells, NK cells, and antigen presenting cells, in subjects suffering from a condition resulting from or exacerbated by ICER-mediated inhbition of immune cell activity.

In addition, the above procedures may be used to treat conditions resulting from or exacerbated by elevated levels of immune cell activity. In such procedures, a vector encoding one or more ICER isoforms or an agent which increases ICER acitivity is introduced into an individual suffering from such a condition as described above.

Agents which reduce or increase ICER activity may also be used as reagents to study the molecular mechanisms and pathways which govern immune cell activity. For example, many agents besides cAMP agonists inhibit cells of the immune system, including IL-10 and glucocorticoids (see above). In addition, tumor cells and infectious pathogens may inhibit cells of the immune system through (1) production of putative inhibitory factors such as prostaglandins and IL-10 (see above); (2) induction of putative inhibitory factors in host cells (see above); (3) production of currently unidentified factors; (4) induction of currently unidentified factors. Thus, agents which reduce the activity of ICER may be introduced into immune cells and to determine whether they reduce or abrogate the ability of the above agents to inhibit immune cell activity. Alternatively, agents which increase ICER activity may be introduced into immune cells and their ability to reduce or prevent further inhibition of immune cell activity may be measured.

For example, *in vitro* or *in vivo* analyses such as those described above may be performed to determine whether the inhibitory effects of such agents as rIL-10, glucocorticoids, or tumor-derived materials on cells of the immune system, such as T cells, B cells, NK cells, and APCs are also blocked by ICER antisense nucleic acids or ICER ribozymes. Cells treated with rIL-10, glucocorticoids, or tumor derived materials are loaded wih ICER antisense nucleic acids or ribozymes or designed to stably express ICER antisense nucleic acids or ribozymes as described above.

The level of inhibition of immune cell activity is measured in cells receiving the agents and compared to that observed in cells receiving control nucleic acids to identify factors which inhibit immune cell activity via ICER.

The protein(s) responsible for sustained ICER expression under conditions in which cytokine expression is inhibited by ICER, hereinafter referred to as RINSR (Repressor(s) of ICER Negative Self-regulation (RINSR), or nucleic acids encoding the RINSR protein(s) are isolated as follows.

Example 40

Detection and Isolation of RINSR

Standardized techniques such as Differential Display, Serial Analysis of Gene Expression (SAGE), and/or Gene Calling™ (Curagen Corp.) are used to identify mRNAs/proteins uniquely synthesized in response to combined stimuli (e.g., forskolin plus ionomycin) which result in sustained expression of ICER. mRNAs or proteins synthesized in response to such combined stimuli but not to either stimulus are likely to constitute factors responsible for repressing ICER negative self-regulation. The genes encoding such proteins are cloned using standard techniques and the protein and mRNA sequences are examined to determine whether they are identical to putative mRNAs/proteins, or whether they represent previously unidentified regulatory elements. Antisense intervention therapies in which RINSR antisense is used to block sustained RINSR expression are then designed.

Example 41

Reduction of ICER Activity Using RINSR Antisense

As described above, ICER autoregulation may be inhibited by RINSR, a protein(s) which prevents ICER from inhibiting its own transcription. Following identification and cloning of RINSR as described above, cells such as T cells, B cells, NK cells, monocytes, dendritic cells, or other APCs are treated with cAMP agonists and either RINSR antisense or control sense nucleic acids as described above. ICER-mediated inhibition of immune cell activity is reduced or prevented in cells receiving RINSR antisense but not in cells receiving control nucleic acids.

ICER antisense may be used to reduce or prevent ICER-mediated inhibition of immune cell activity in subjects in which immune cell activity is repressed by tumors or pathogenic agents. Representative examples of the use of ICER antisense as a therapeutic for reducing or preventing ICER-mediated inhibition of immune cell activity are described below. However, it will be appreciated that ICER antisense may be used as a therapeutic to treat any condition in which immune cell activity is inhibited by ICER.

It will be appreciated that strategies other than ICER antisense or ICER ribozymes may be employed to reduce or prevent ICER-mediated inhibition of immune cell activity. Such strategies include any method of blocking or reducing transcription of the ICER gene, translation of the ICER mRNA, or the activity of the ICER protein. For example, in one such approach peptides which block or reduce the activity of ICER or RINSR may be employed.

Although this invention has been described in terms of certain preferred embodiments, other embodiments which will be apparent to those of ordinary skill in the art in view of the disclosure herein are also within the scope of this

invention. All documents cited herein, including the references listed below, are incorporated herein by reference in their entirety.

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WHAT IS CLAIMED IS:

1. An agent which decreases the level of ICER expression for use in increasing the activity of an immune cell.

2. The agent of Claim 1, wherein siad agent comprises a nucleic acid.

3. The agent of Claim 1, wherein said nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

4. The agent of Claim 1, wherein said nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

5. Use of an agent which decreases the level of ICER expression in the preparation of a medicament for increasing the activity of an immune cell.

6. The use of Claim 5, wherein said agent comprises a nucleic acid.

7. The use of Claim 5, wherein said nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

8. The use of Claim 5, wherein said nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

9. The use of Claim 5, wherein said medicament is for the treatment of cancer.

10. The use of Claim 5, wherein said medicament is for the treatment of infection with a pathogenic organism.

11. The use of Claim 5, wherein said immune cell is selected from the group consisting of T cells, B cells, NK cells, monocytes, dendritic cells and antigen presenting cells.

12. The use of Claim 11, wherein said immune cell is a T cell.

13. A ribozyme comprising at least one catalytic sequence having endonuclease activity and at least one targetting sequence, said at least one targetting sequence being complementary to a sequence present in at least one isoform of ICER mRNA.

14. An immune cell into which an agent capable of decreasing the expression of ICER has been introduced.

15. The immune cell of Claim 14, wherein said agent comprises a nucleic acid.

16. The immune cell of Claim 15, wherein said nucleic acid comprises a ribozyme comprising at least one catalytic sequence having endonuclease activity and at least one targetting sequence, said at least one targetting sequence being complementary to a sequence present in at least one isoform of ICER mRNA therein.

17. The immune cell of Claim 15, wherein said nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

18. The immune cell of Claim 14, wherein said immune cell is selected from the group consisting of T cells, B cells, NK cells, monocytes, dendritic cells and antigen presenting cells.

19. The immune cell of Claim 18, wherein said immune cell is a T cell.

20. A method for increasing the activity of an immune cell comprising decreasing the level of ICER expression in said immune cell.

21. The method of Claim 20, wherein the level of ICER expression is decreased by introducing a nucleic acid which inhibits ICER expression into said immune cell.

22. The method of Claim 21, wherein said nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

23. The method of Claim 21, wherein said nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

24. The method of Claim 21, wherein said immune cell is selected from the group consisting of T cells, B cells, NK cells, monocytes, dendritic cells and antigen presenting cells.

25. A method of increasing the activity of immune cells in an individual comprising the steps of:

removing said immune cells from said individual;

introducing an agent which inhibits ICER expression into said immune cells;

and reintroducing said immune cells into said individual.

26. The method of Claim 25, wherein said agent comprises a nucleic acid.

27. The method of Claim 26, wherein said nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

28. The method of Claim 26, wherein said nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

29. The method of Claim 25, wherein said immune cell is selected from the group consisting of T cells, B cells, NK cells, and antigen presenting cells.

30. The method of Claim 29, wherein said immune cell is a T cell.

31. The method of Claim 25, wherein said individual suffers from a condition which reduces immune cell activity.

32. The method of Claim 25, wherein said individual is infected with a pathogenic organism.

33. The method of Claim 25, wherein said individual suffers from cancer.

34. A method of increasing the activity of immune cells in an individual comprising :

introducing an agent which inhibits ICER expression into immune cells in said individual.

35. The method of Claim 34, wherein said agent comprises a nucleic acid.

36. The method of Claim 35, wherein said nucleic acid comprises a ribozyme capable of cleaving at least one site in at least one isoform of ICER mRNA.

37. The method of Claim 35, wherein said nucleic acid comprises an antisense nucleic acid comprising a sequence complementary to at least a portion of at least one isoform of ICER mRNA.

38. The method of Claim 34, wherein said immune cell is selected from the group consisting of T cells, B cells, NK cells, and antigen presenting cells.

39. The method of Claim 38, wherein said immune cell is a T cell.

40. The method of Claim 34, wherein said individual suffers from a condition which reduces immune cell activity.

41. The method of Claim 34, wherein said individual is infected with a pathogenic organism.

42. The method of Claim 34, wherein said individual suffers form cancer.

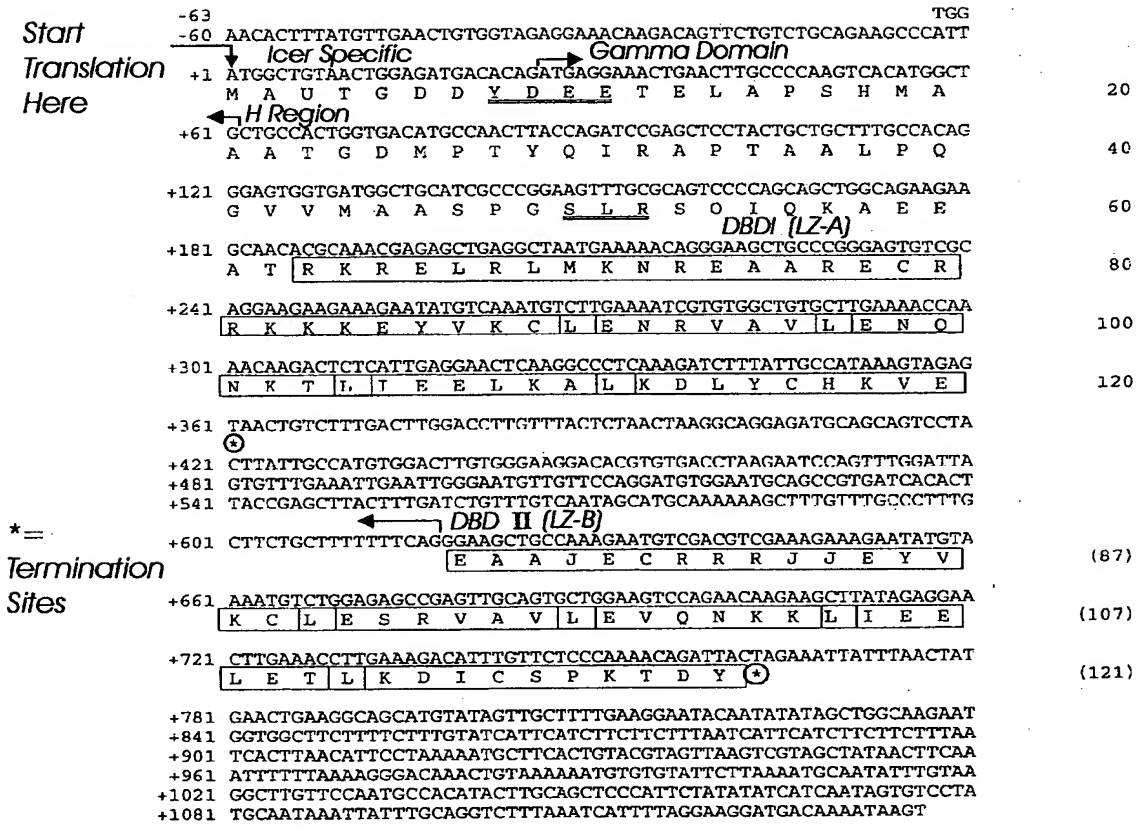
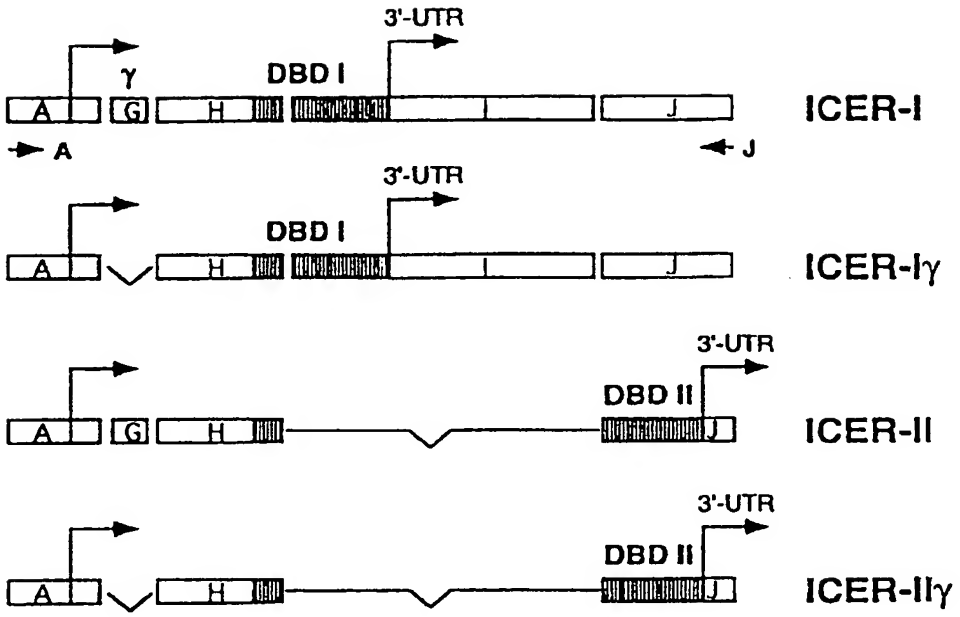

Start Translation Here
Icer Specific
Gamma Domain
H Region
DBDI (LZ-A)
DBD II (LZ-B)
*= Termination Sites

FIG. 1



Top, Modules composing ICER transcript isoforms and positions of primers used for RT-PCR amplification of ICER transcripts from cultured human ES cells are shown. *Below*, All four known ICER isoforms were isolated (27, 44). The γ-isoforms are lacking the small exon γ. In the ICER-II forms module I is absent, leading to expression of DBD II and utilization of the second stop codon.

FIG.2

ICER mRNAs are persistently expressed by CD4 T-cells whose ionomycin-induced proliferation is inhibited by forskolin

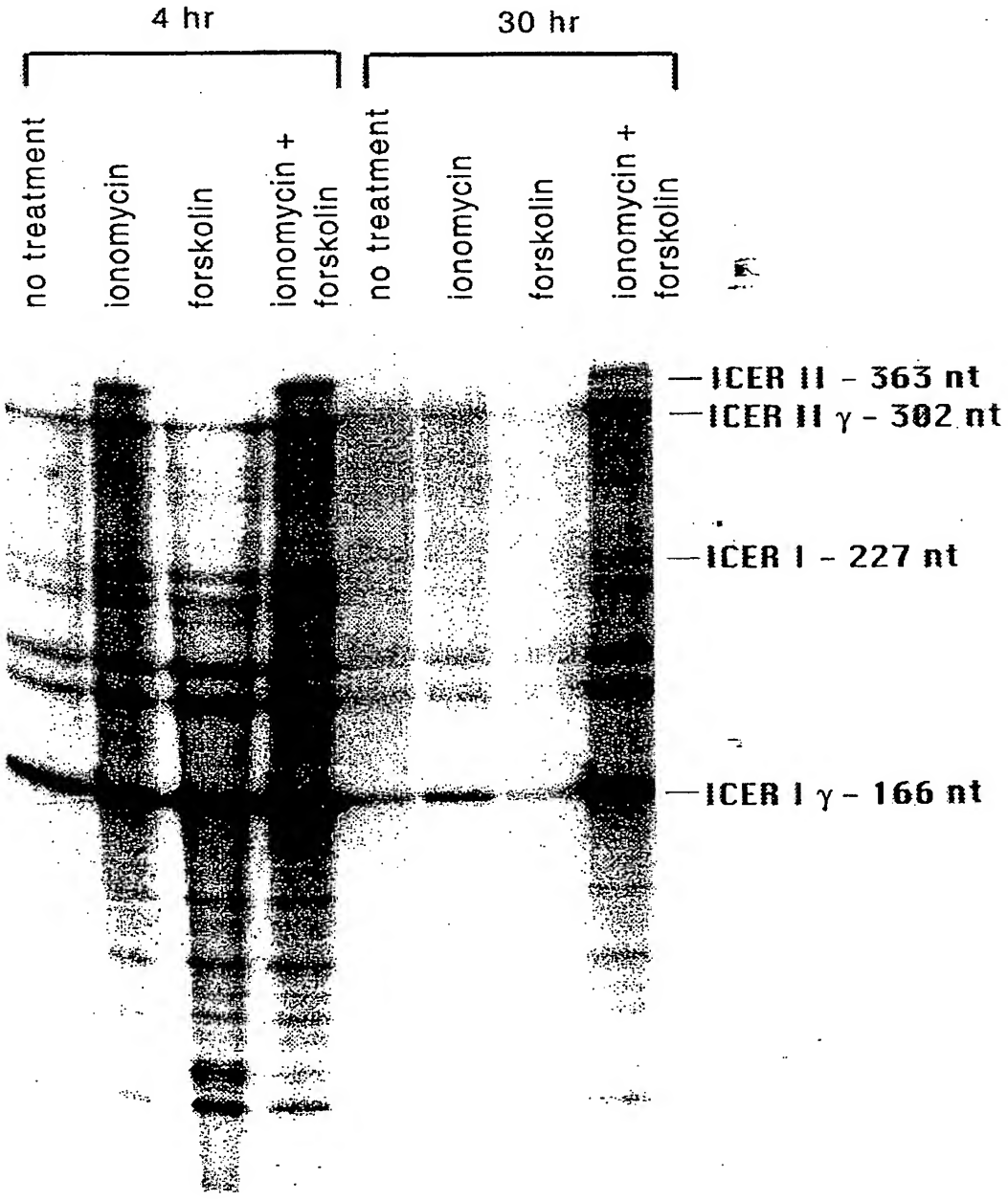


FIG. 3

Multiple stimuli induce transient ICER mRNA expression in monocytes after 4 hrs

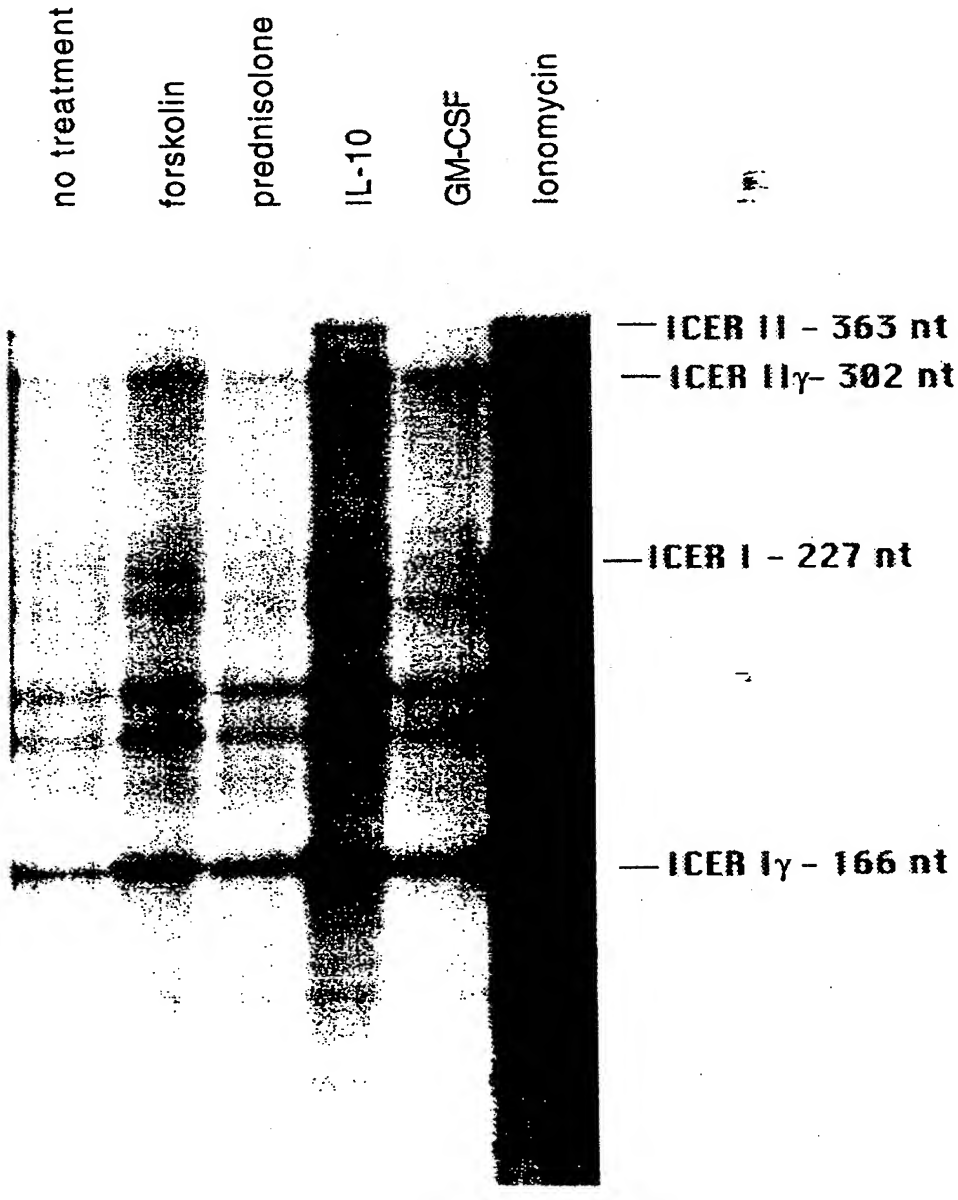


FIG.4

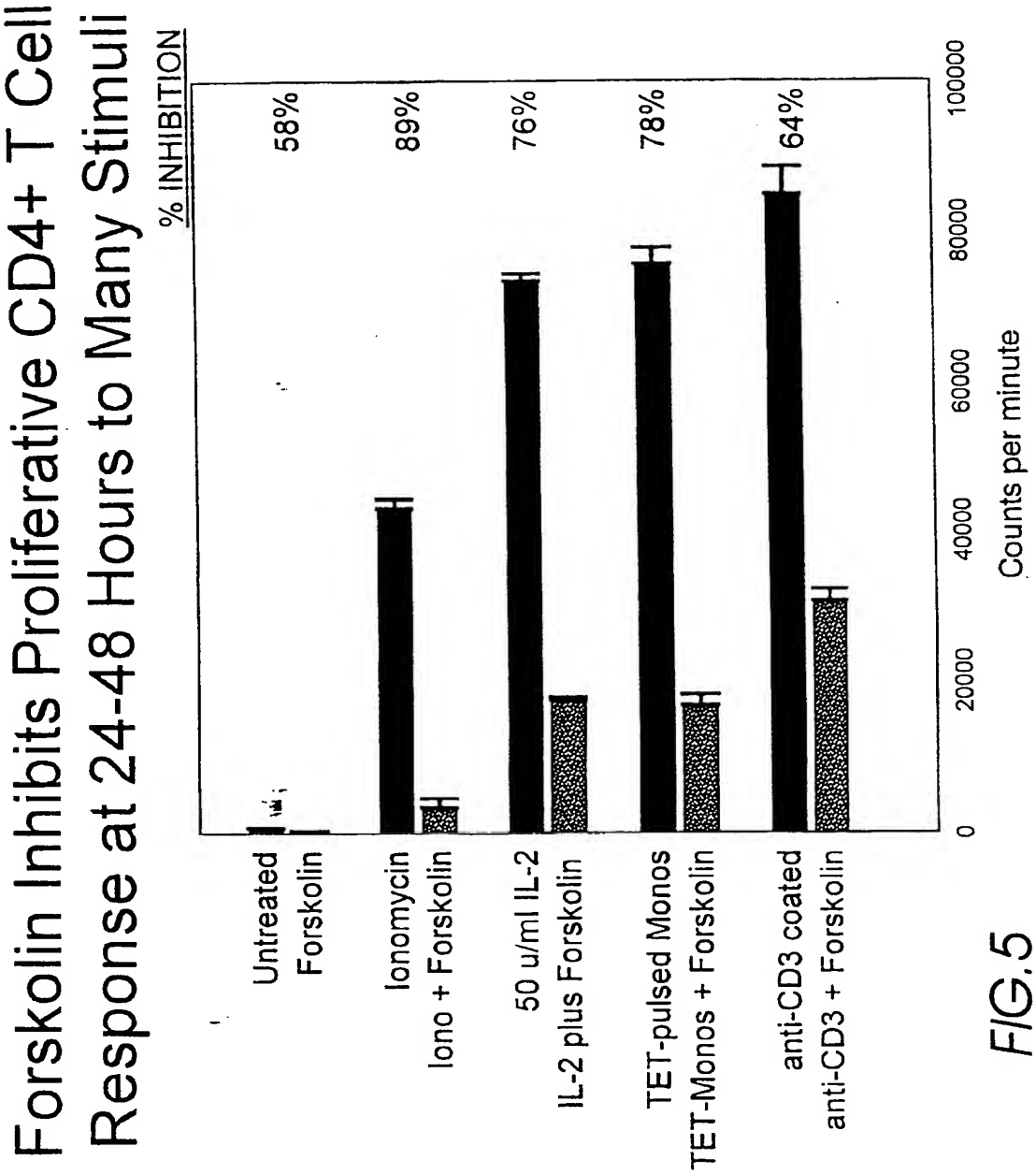

Forskolin Inhibits Proliferative CD4+ T Cell Response at 24-48 Hours to Many Stimuli
% INHIBITION
58%
89%
76%
78%
64%
Untreated
Forskolin
Ionomycin
Iono + Forskolin
50 u/ml IL-2
IL-2 plus Forskolin
TET-pulsed Monos
TET-Monos + Forskolin
anti-CD3 coated
anti-CD3 + Forskolin
0
20000
40000
60000
80000
100000
Counts per minute

FIG.5

ICER mRNAs are persistently expressed by monocytes whose ionophore-induced differentiation is inhibited by forskolin

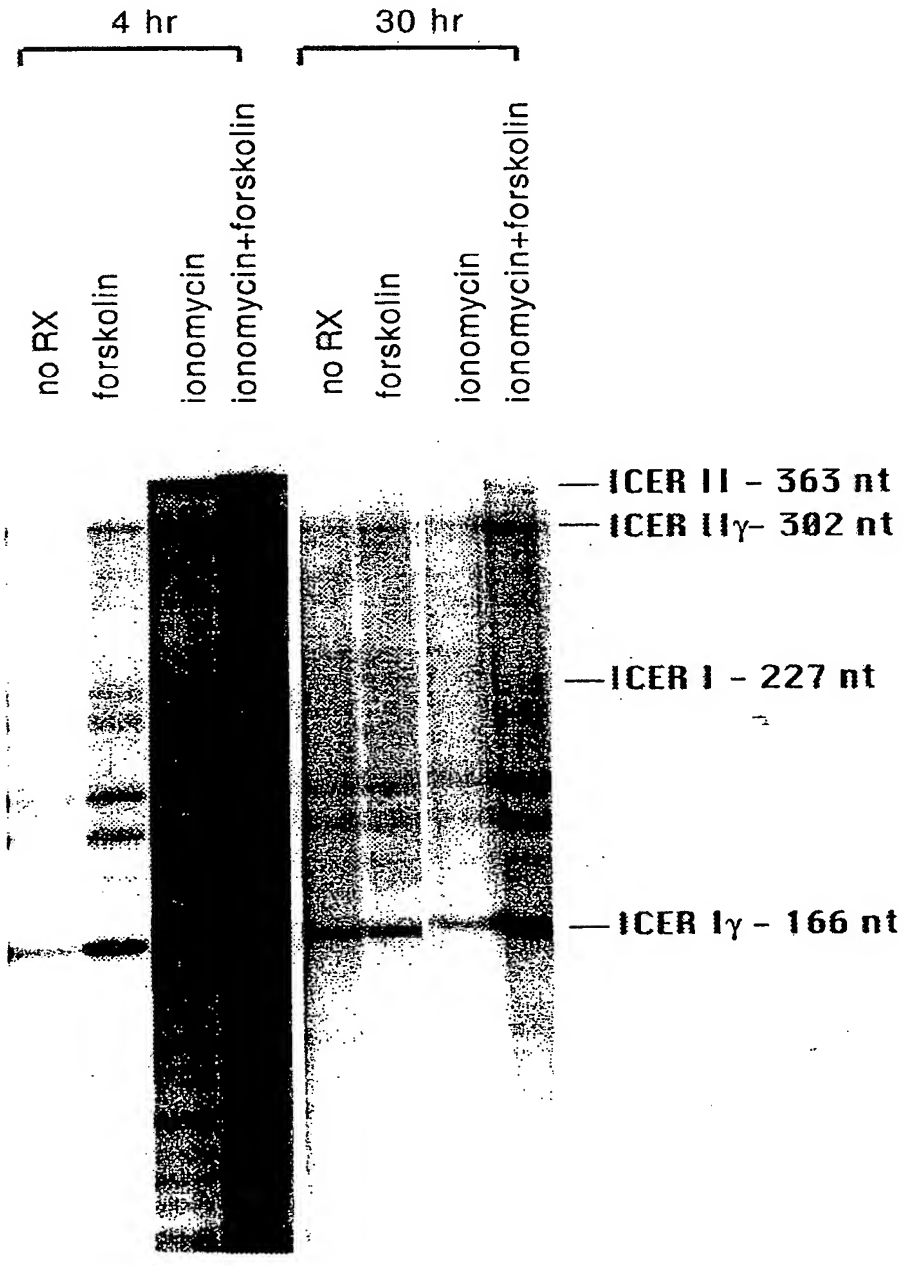


FIG.6

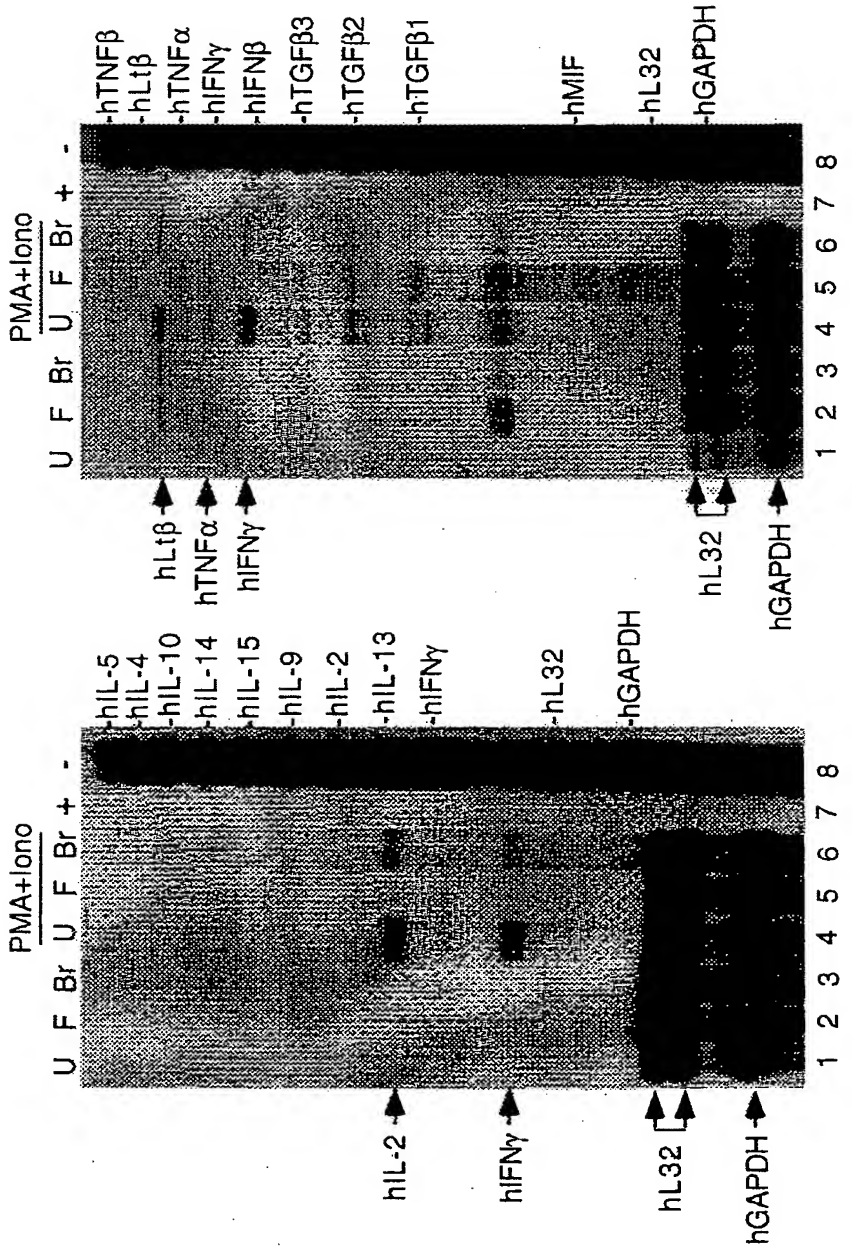

PMA+Iono
U F Br U F Br + -
1 2 3 4 5 6 7 8
-hIL-5
-hIL-4
-hIL-10
-hIL-14
-hIL-15
-hIL-9
-hIL-2
-hIL-13
-hIFNγ
-hL32
-hGAPDH
hIL-2
hIFNγ
hL32
hGAPDH
PMA+Iono
U F Br U F Br + -
1 2 3 4 5 6 7 8
-hTNFβ
-hLtβ
-hTNFα
-hIFNγ
-hIFNβ
-hTGFβ3
-hTGFβ2
-hTGFβ1
-hMIF
-hL32
-hGAPDH
hLtβ
hTNFα
hIFNγ
hL32
hGAPDH

FIG. 7A

FIG. 7B

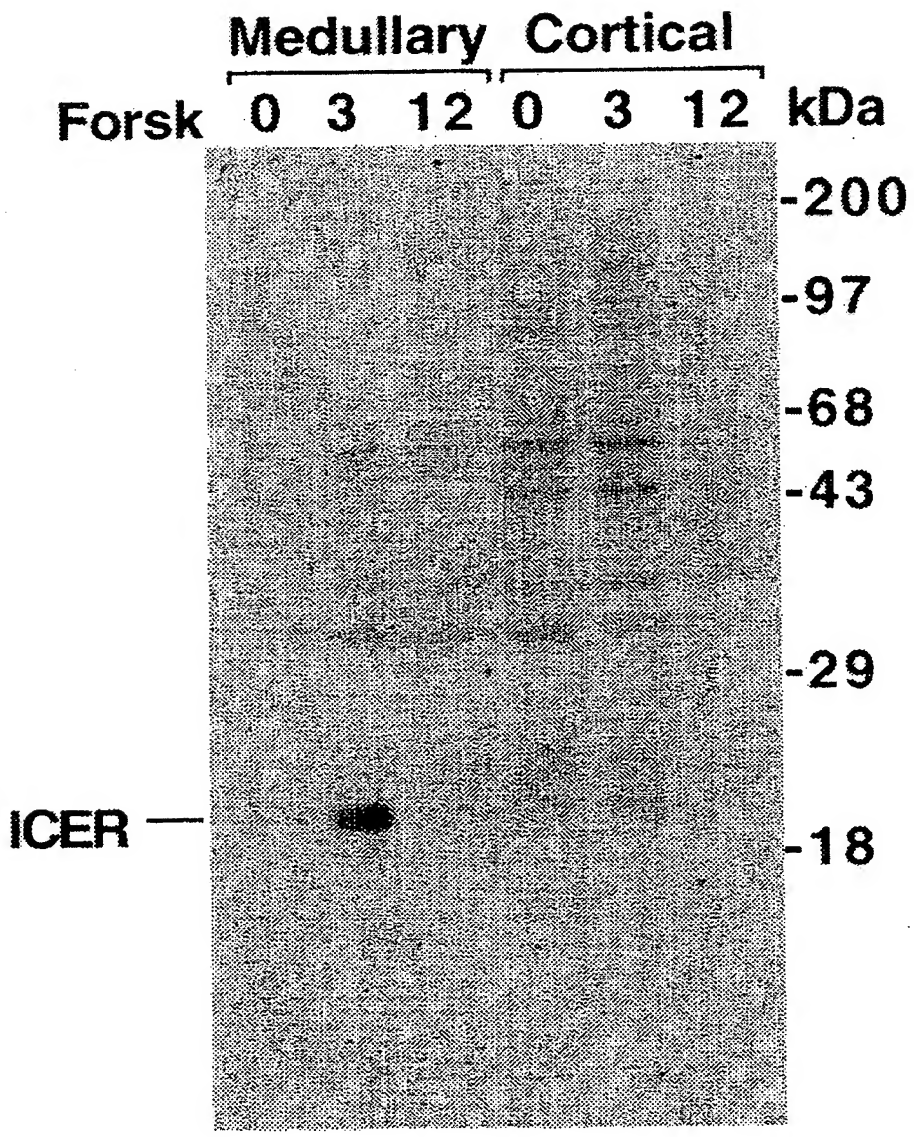

Medullary
Cortical
Forsk
0
3
12
0
3
12
kDa
-200
-97
-68
-43
-29
-18
ICER

FIG. 7C

FIG. 8

	IL-2	NFAT	AP-1
SEQ ID NO.18	-45	TGGAAAAAT	
SEQ ID NO.19	-90	TTGAAAATATGTGTAATA	
SEQ ID NO.20	-135	AGGAAAAACAAAGGTAAT	
SEQ ID NO.21	-160	AGAAATTCCAGAGAGTCA	
SEQ ID NO.22	-280	AGGAAAATTTGTTTCATA	

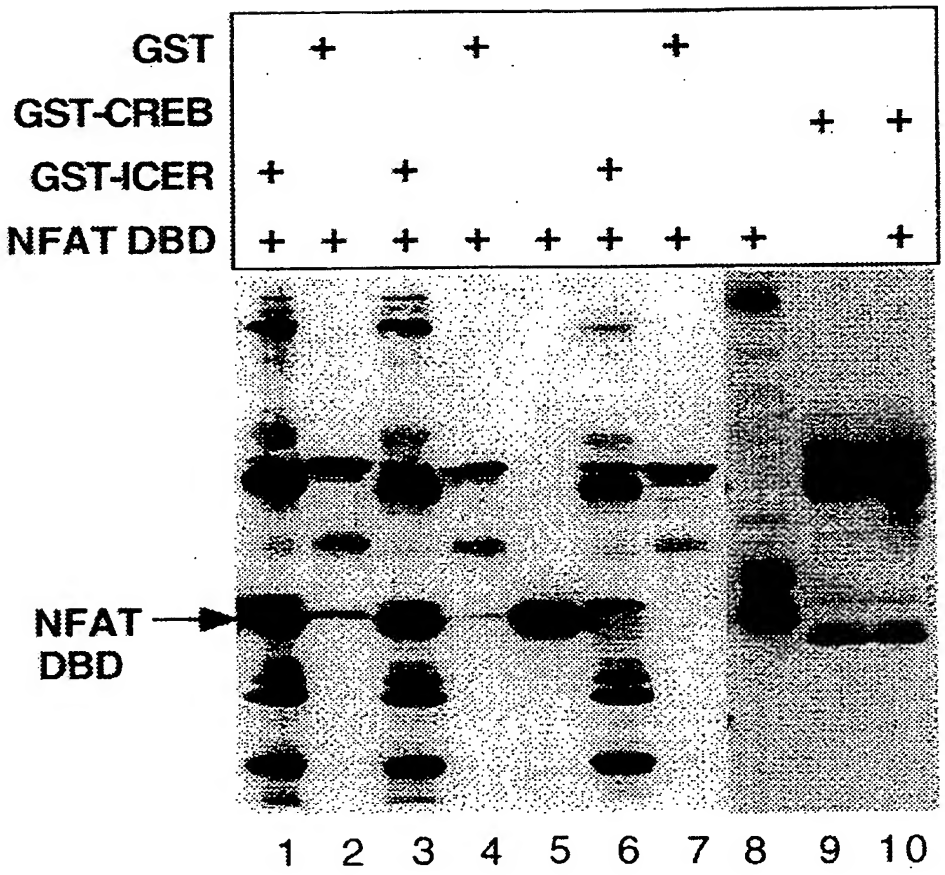
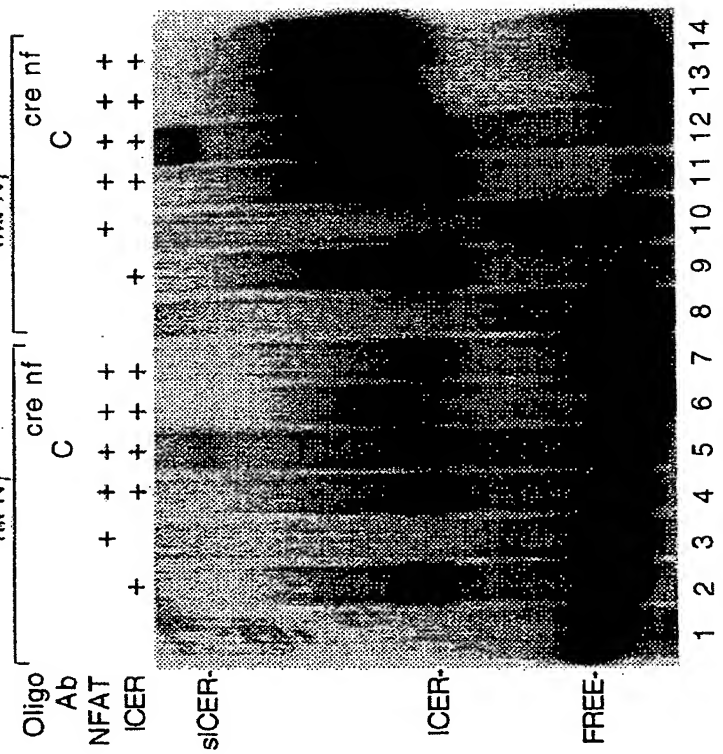


FIG.9

FIG. 10



SEQ ID NO.23 mouse AAA CCA AAA AAA AAC AAC TTG TGA AAA TAC GTA ATC CCG AGG

SEQ ID NO.24 human CTC ATC TTA AAA AAC AAC TTG TGA AAA TAC GTA ATC CTC AGG

** ** ** ** ** *** * *

SEQ ID NO.25 human IL-2(-90) AA AAT ATG TGT AA- TAT GTA AAA C

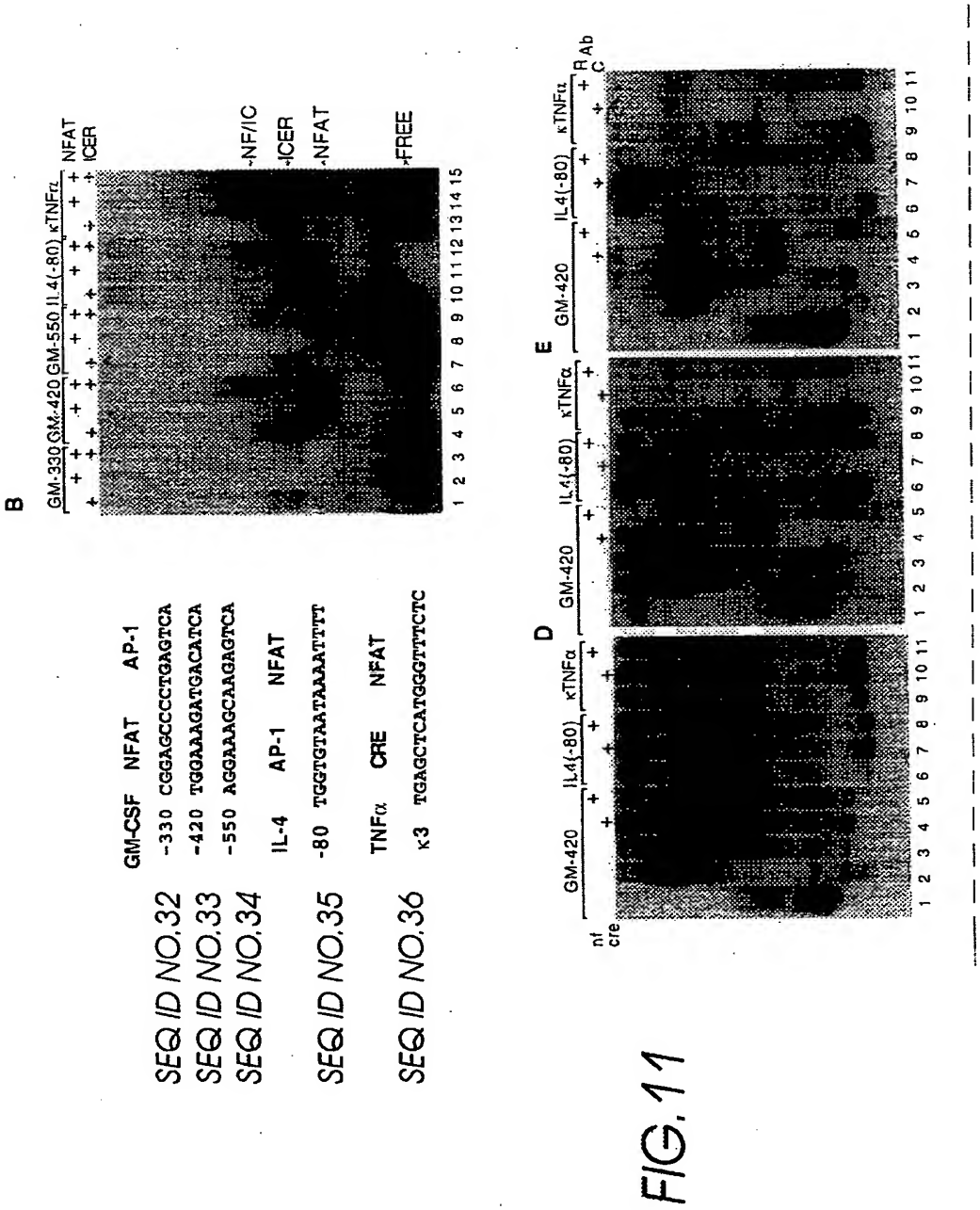

B
GM-330 GM-420 GM-550 IL4(-80) κTNFα
NFAT
ICER
NF/IC
ICER
NFAT
FREE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
GM-CSF NFAT AP-1
SEQ ID NO.32 -330 CGGAGCCCCTGAGTCA
SEQ ID NO.33 -420 TGGAAAGATGACATCA
SEQ ID NO.34 -550 AGGAAAGCAAGAGTCA
IL-4 AP-1 NFAT
SEQ ID NO.35 -80 TGGTGTAATAAAATTTT
TNFα CRE NFAT
SEQ ID NO.36 κ3 TGAGCTCATGGGTTTCTC
D
GM-420 IL4(-80) κTNFα
nt
cre
1 2 3 4 5 6 7 8 9 10 11
GM-420 IL4(-80) κTNFα
1 2 3 4 5 6 7 8 9 10 11
E
GM-420 IL4(-80) κTNFα
RAb
C
1 2 3 4 5 6 7 8 9 10 11

FIG. 11

FIG. 12A

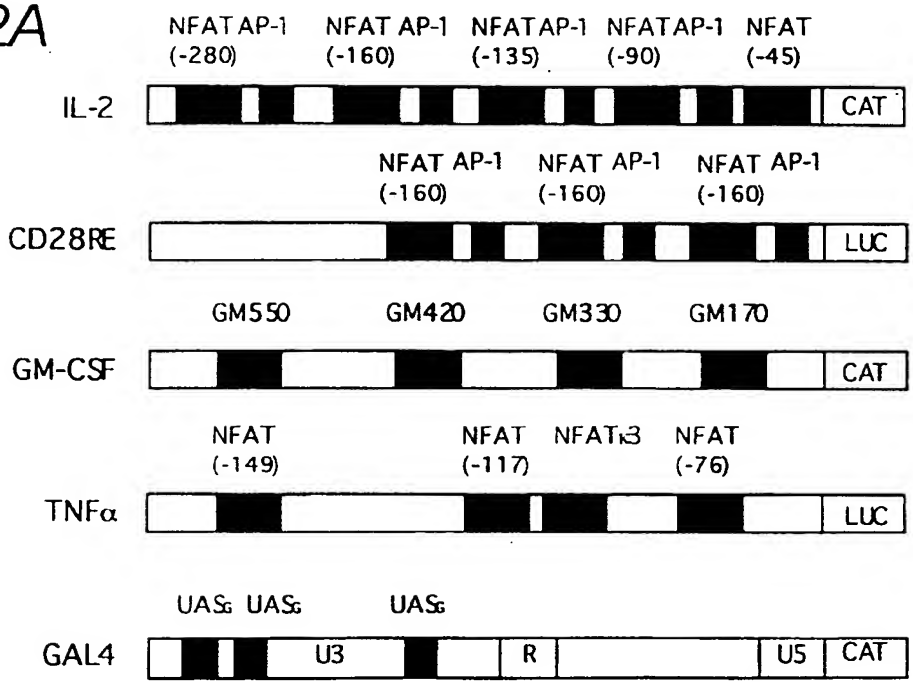
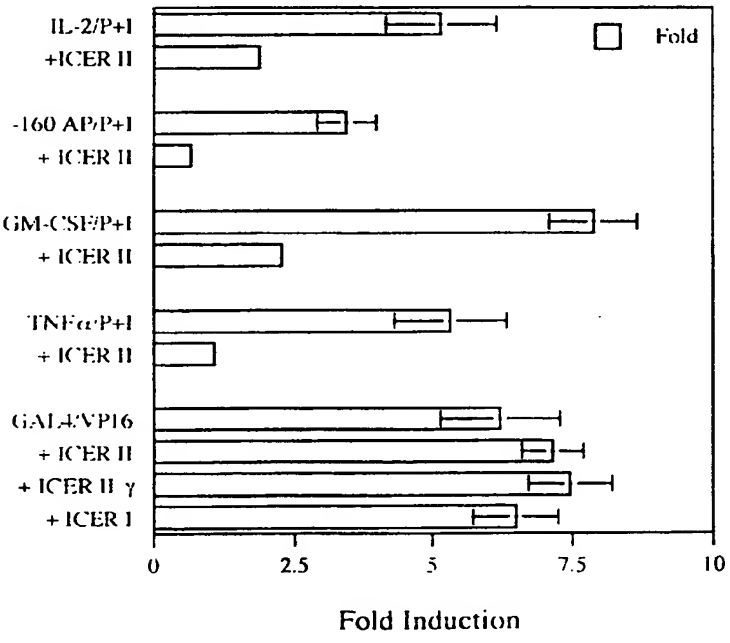
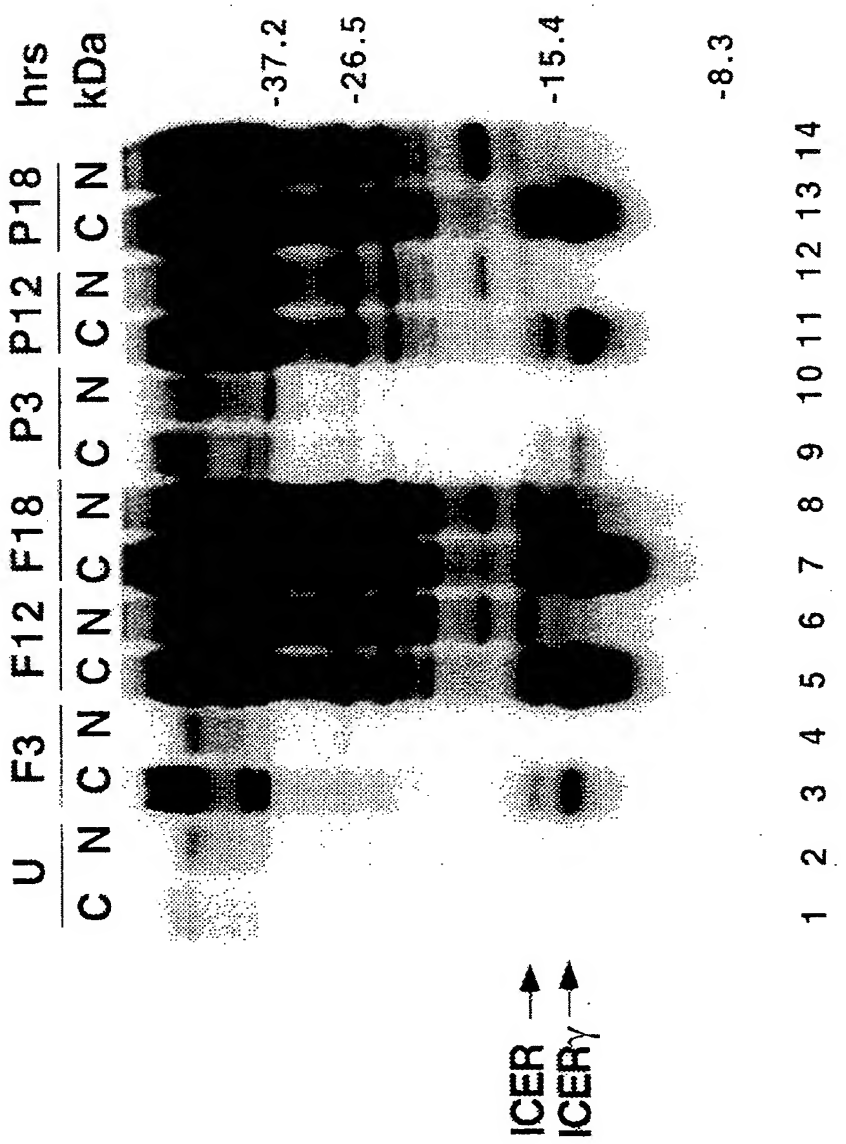


FIG. 12B

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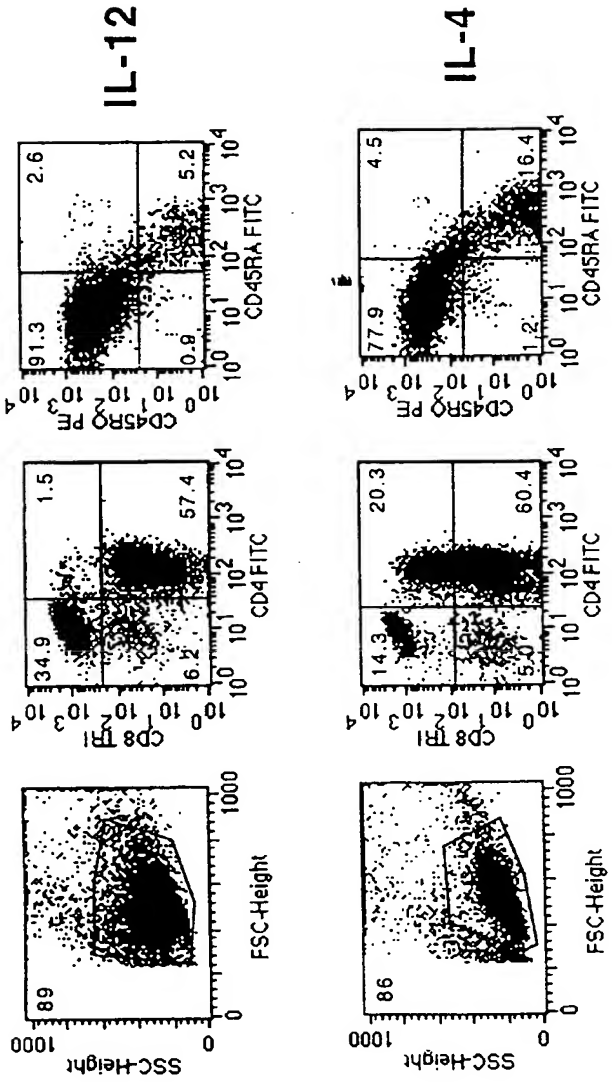
ICER
ICERγ

FIG. 13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34.9
1.5
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20.3
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5.0
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FIG. 14A

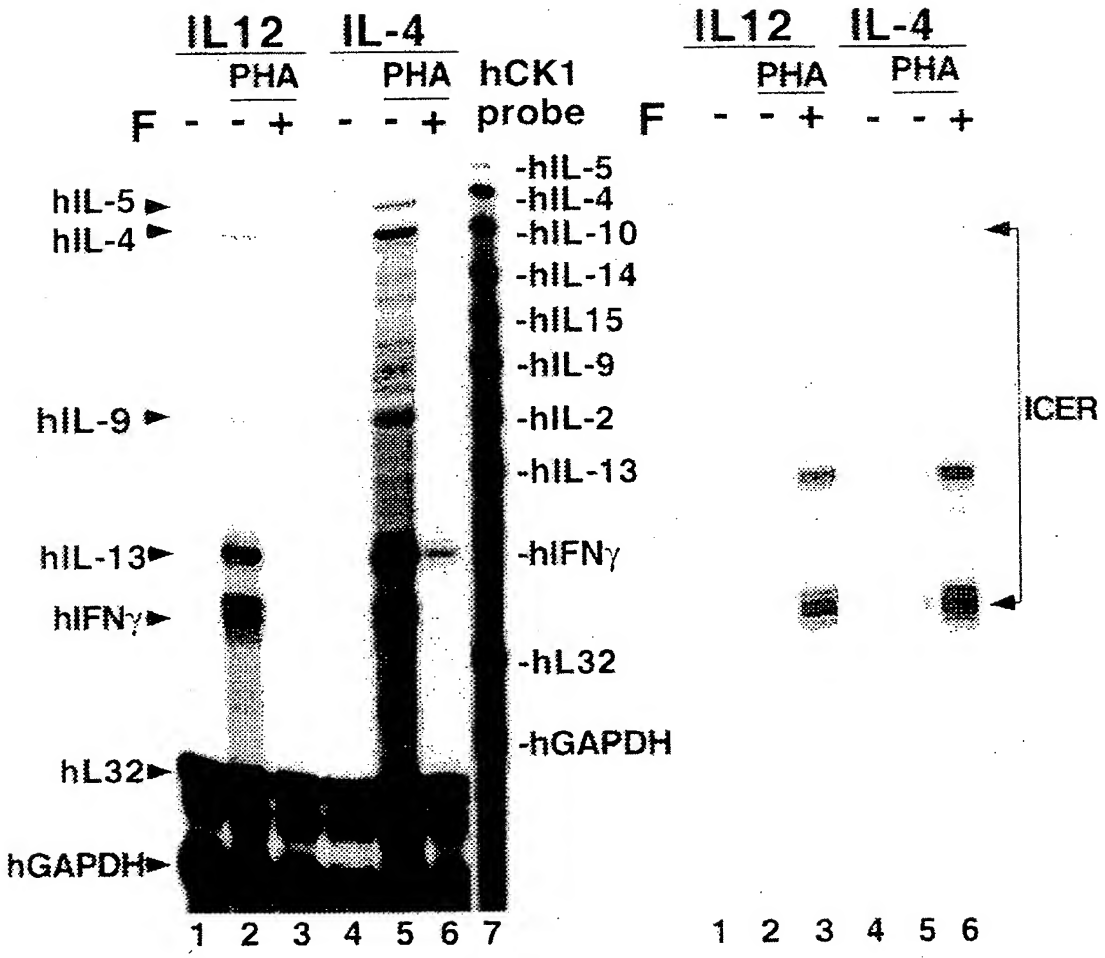

FIG. 14B

FIG. 14C

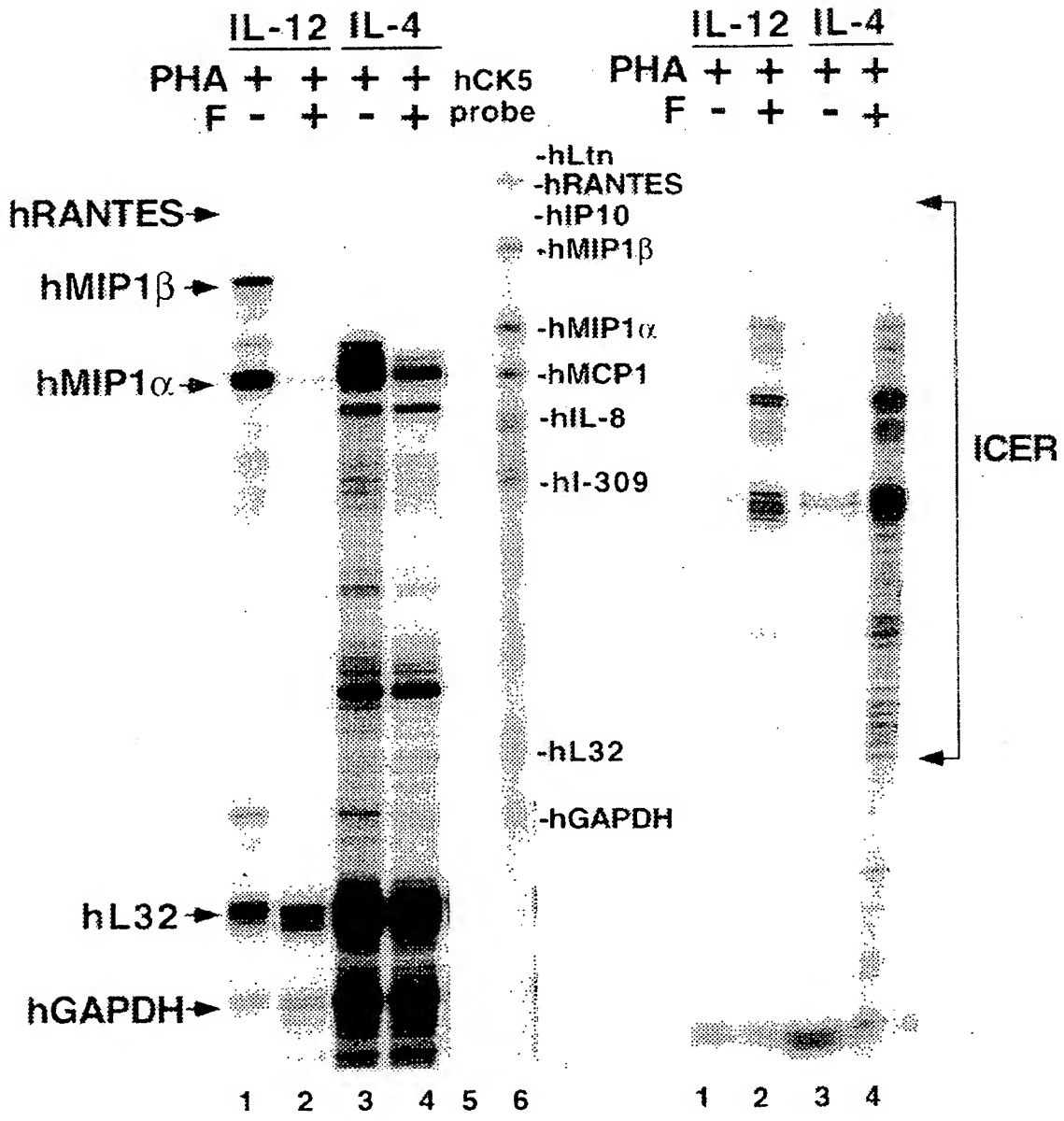

FIG. 14D FIG. 14E

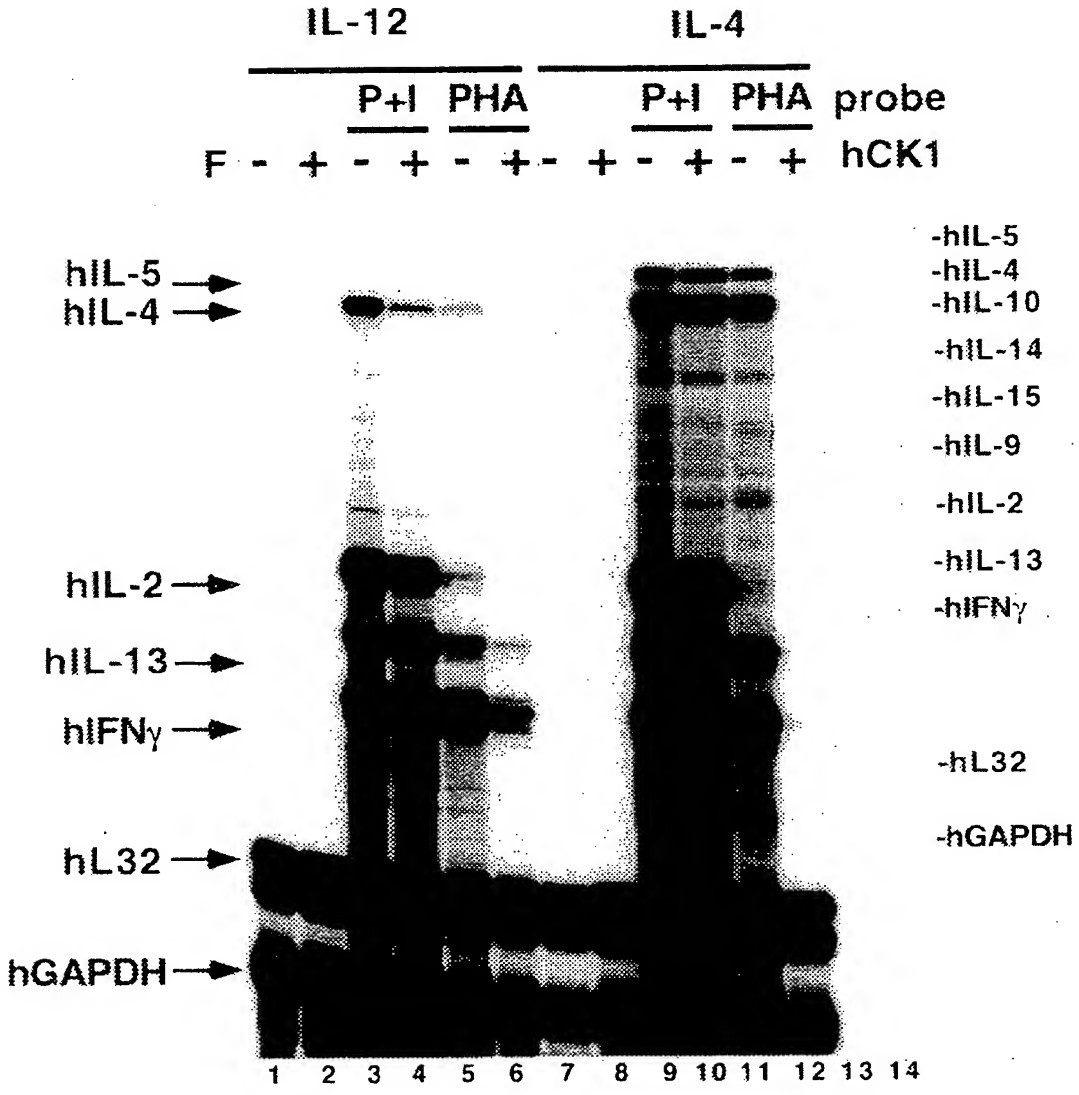
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FIG. 15

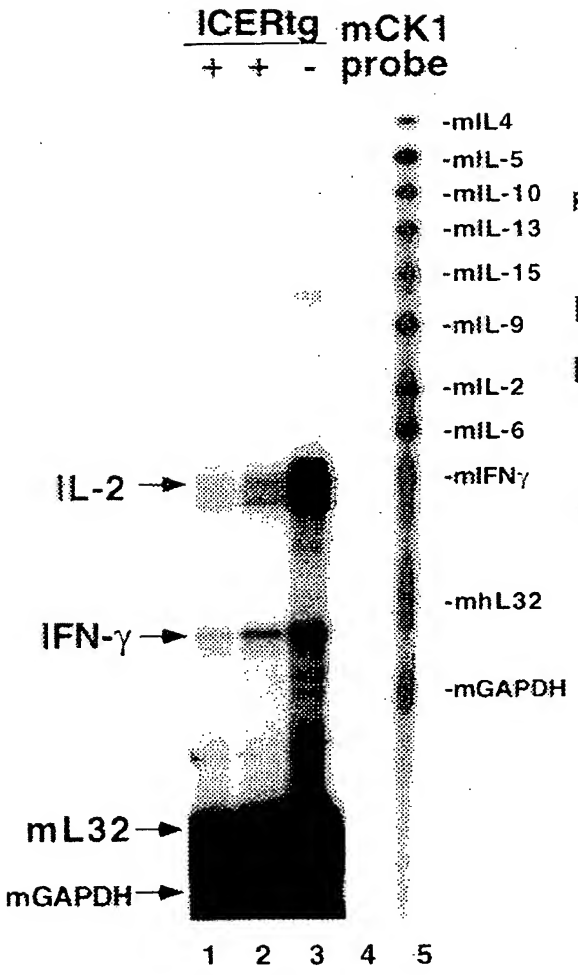

FIG. 16A

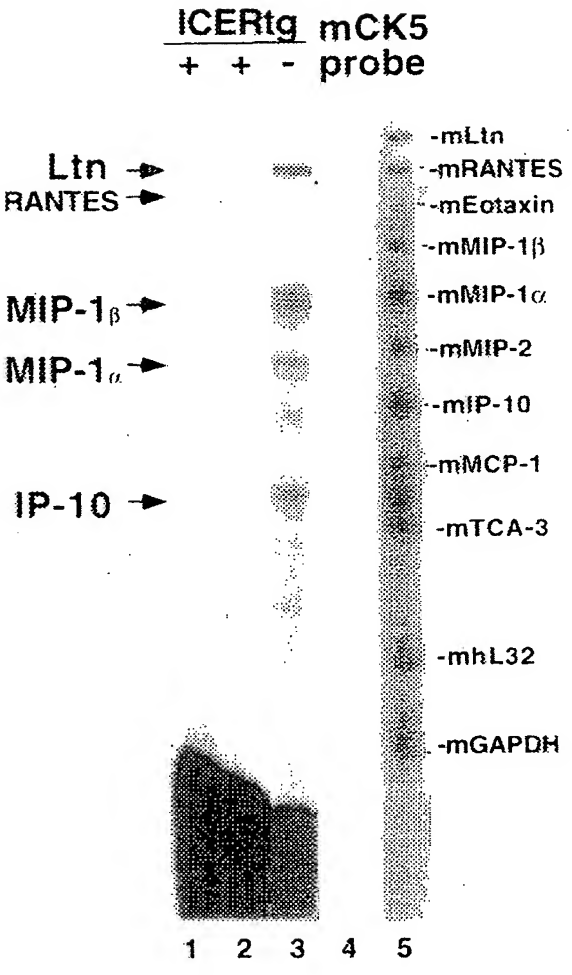

FIG. 16B

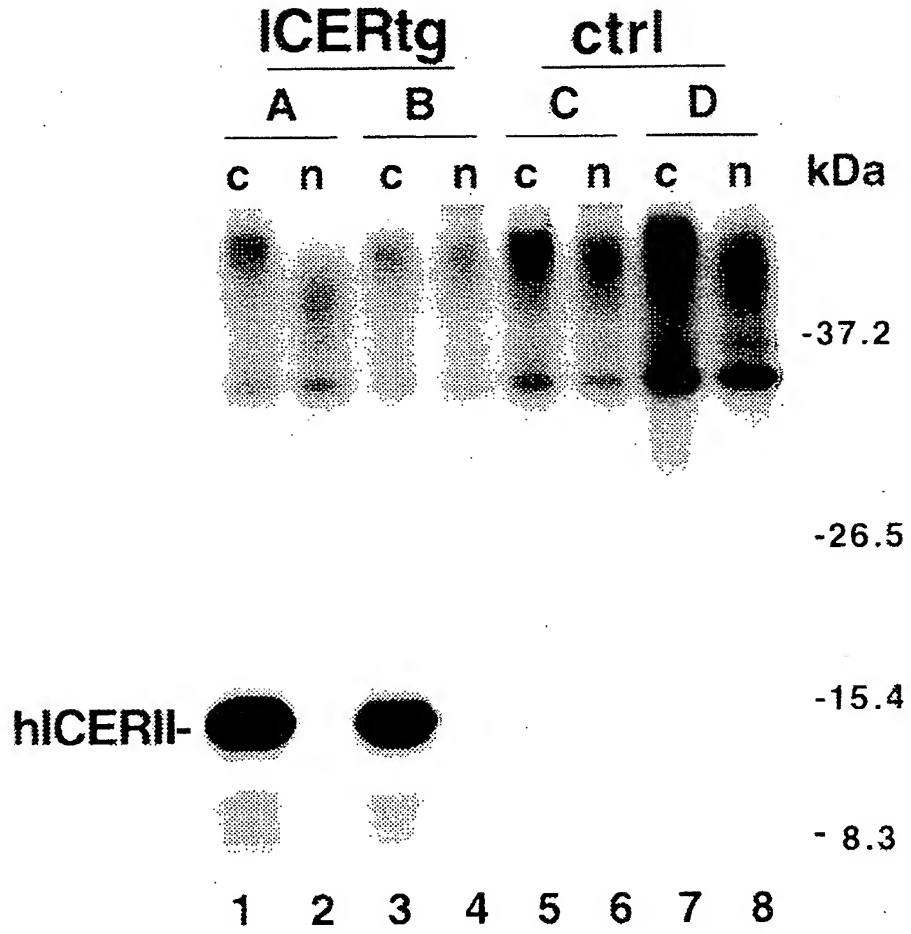
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FIG. 17A

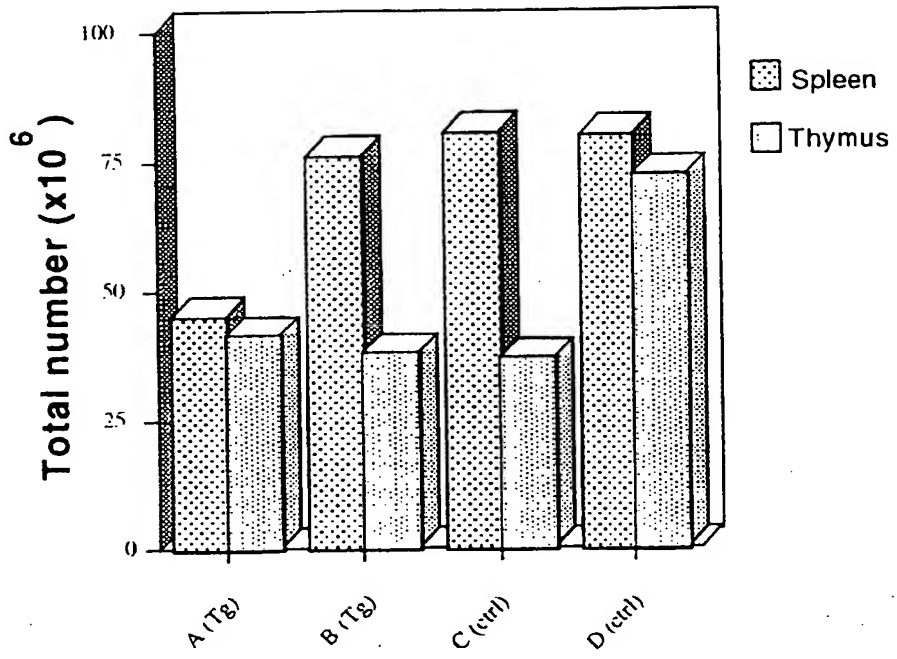


FIG. 17D

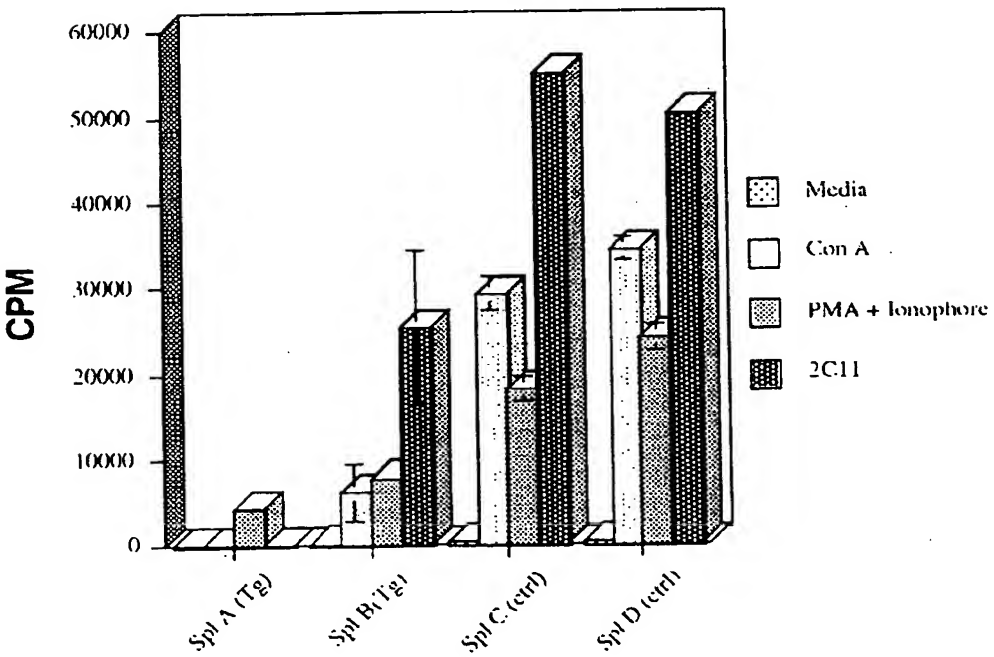
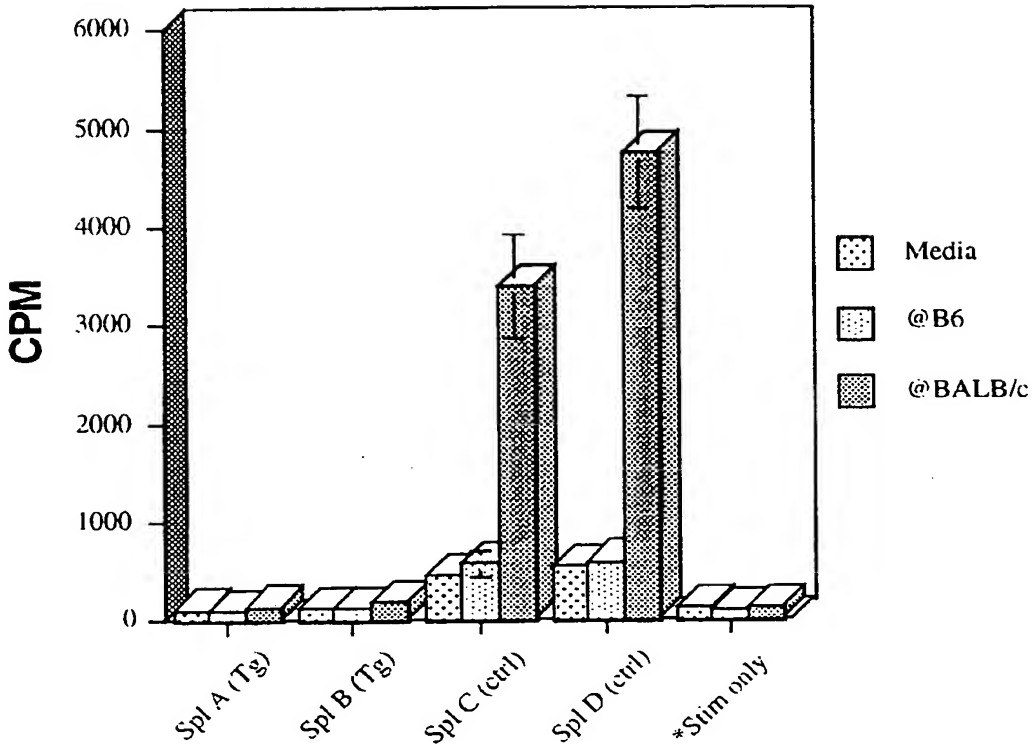


FIG. 17B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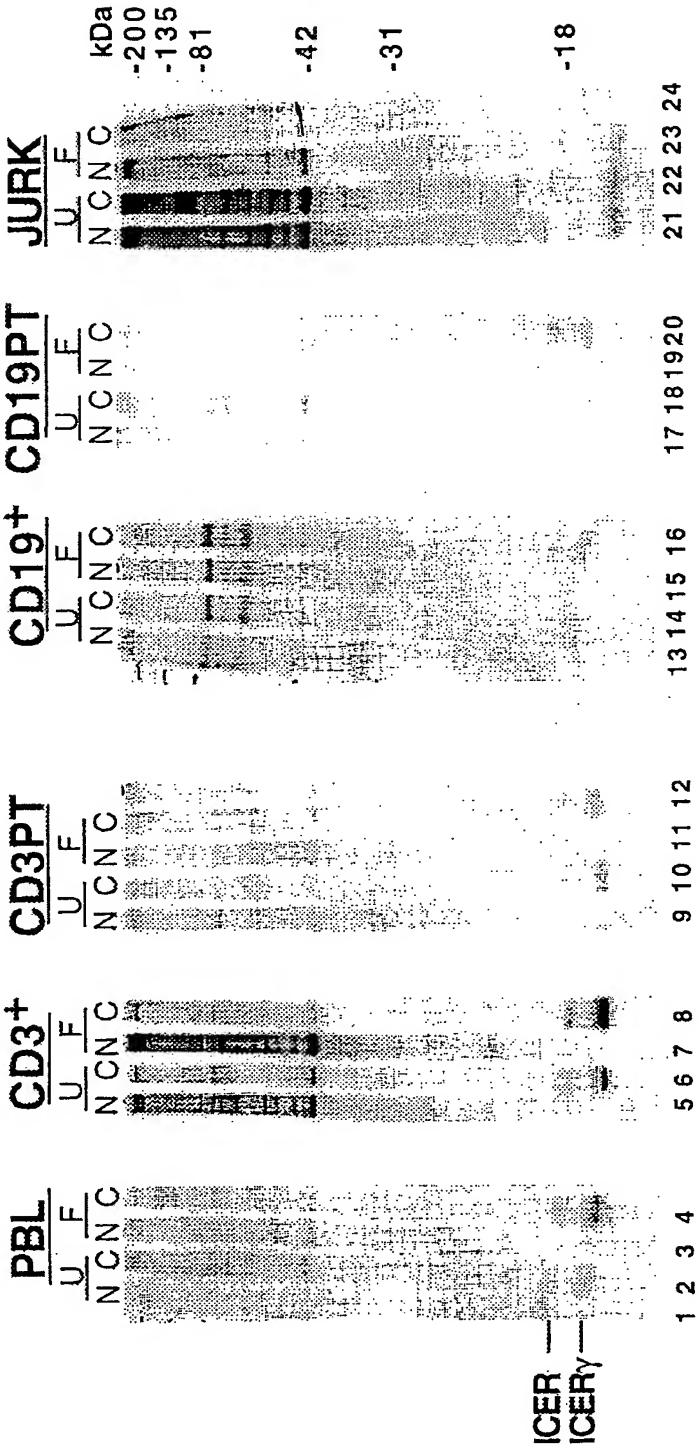
Spl B (Tg)
Spl C (ctrl)
Spl D (ctrl)
*Stim only

FIG. 17C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5 6 7 8
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ICER
ICERγ

FIG. 18

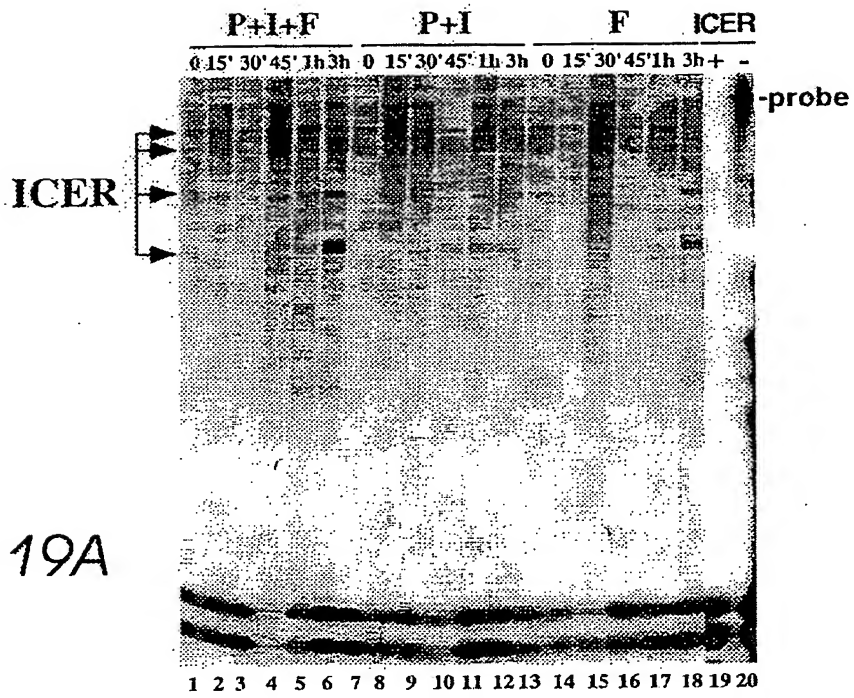

FIG. 19A

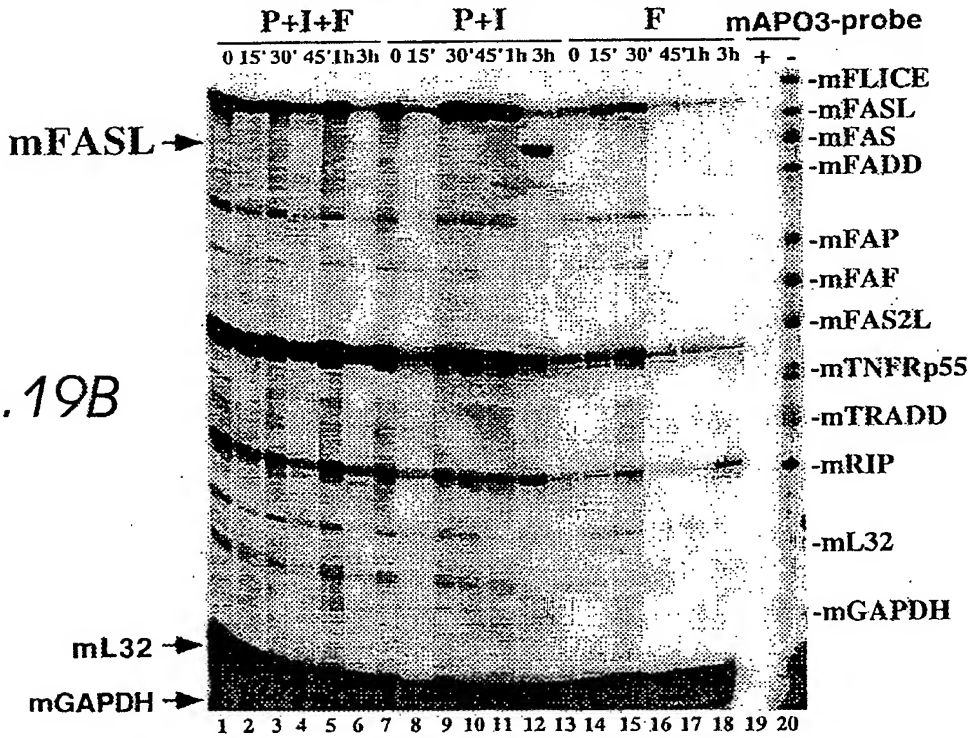

FIG. 19B

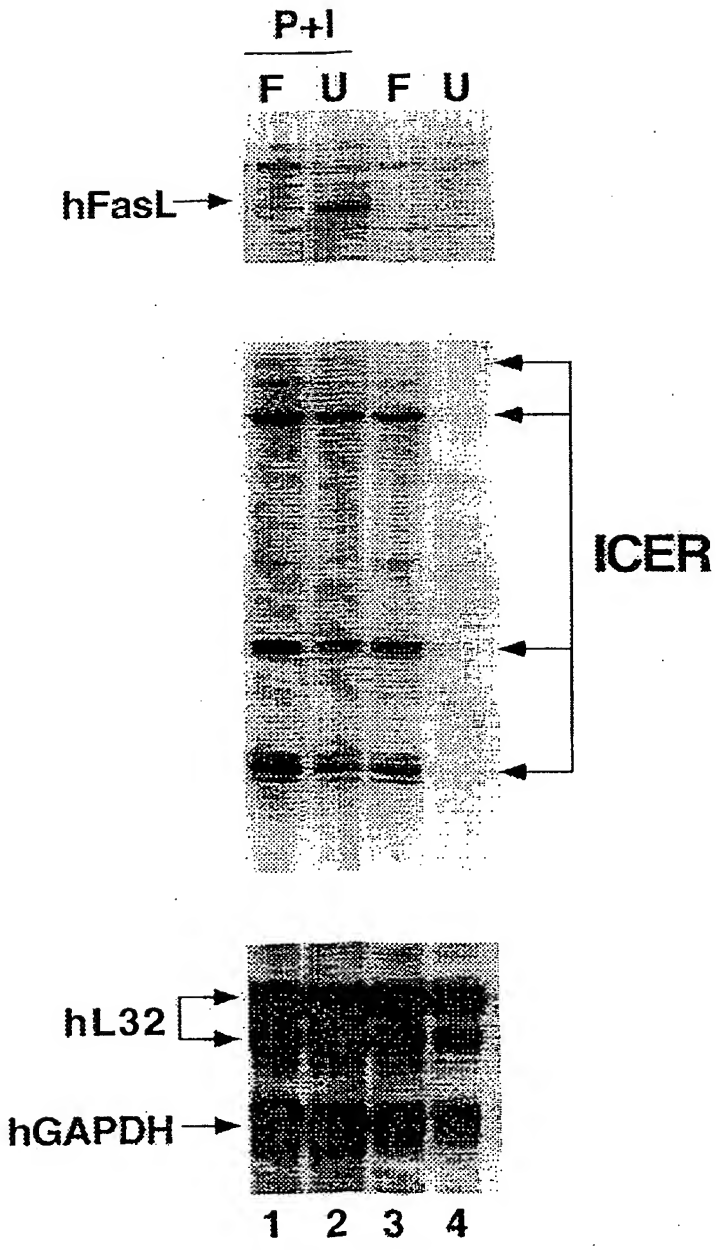

FIG. 20

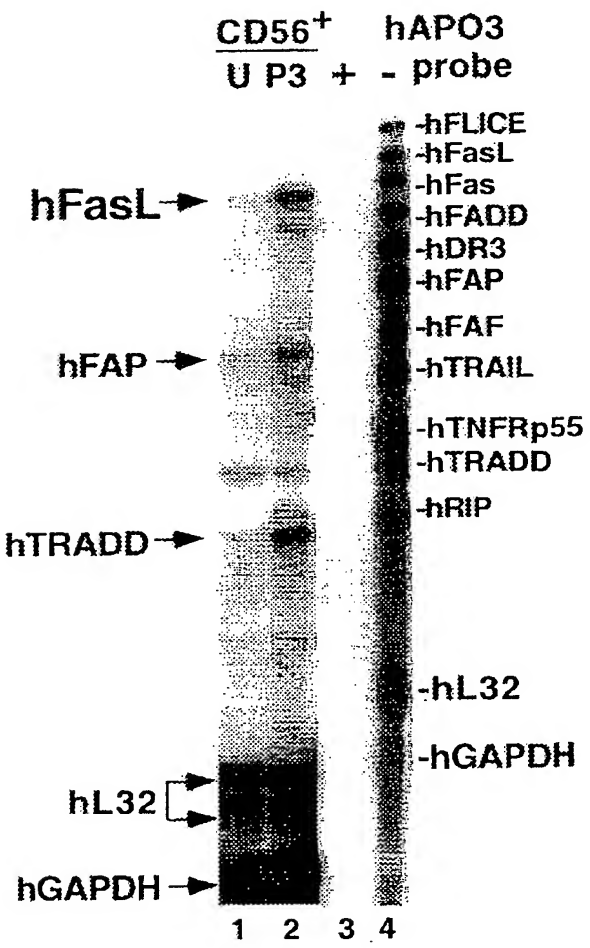

FIG.21A

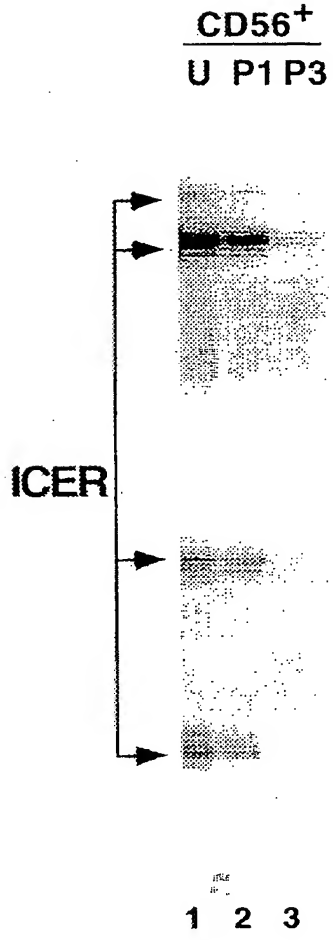

FIG.21B

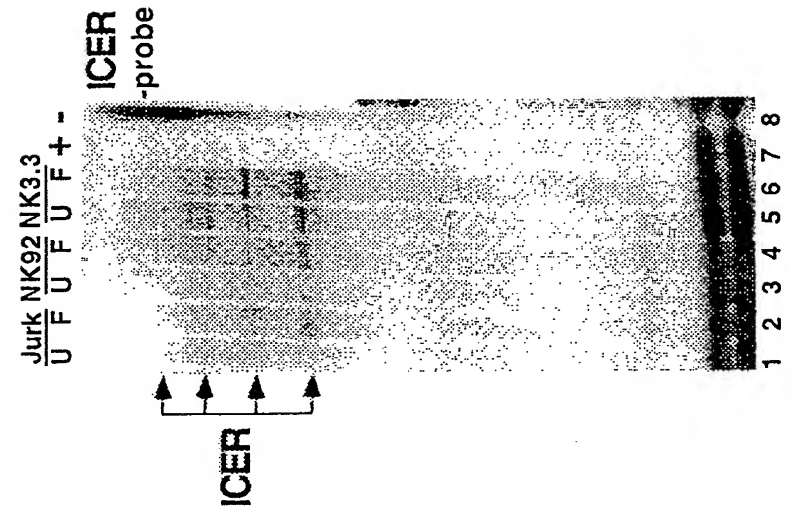

FIG.22B

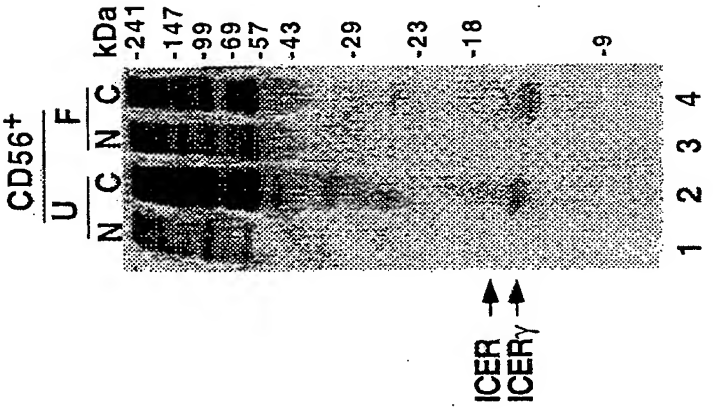

FIG.22A

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SEQ ID NO.27	FasL dist	-275	CGGAAACTT	
SEQ ID NO.28	IL-2(-160)	-160	AAGAAATTCAGAGAGTCA	
SEQ ID NO.29	GM (-420)	-420	TGGAAAGATGACATCAGG	

FIG.23A

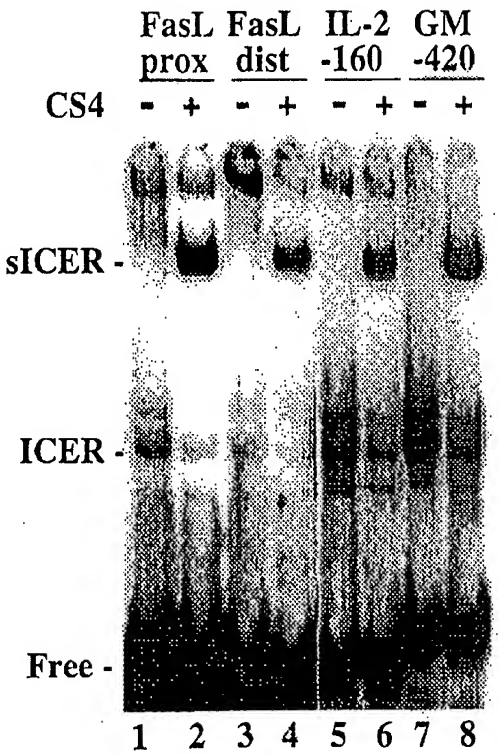

FIG.23B

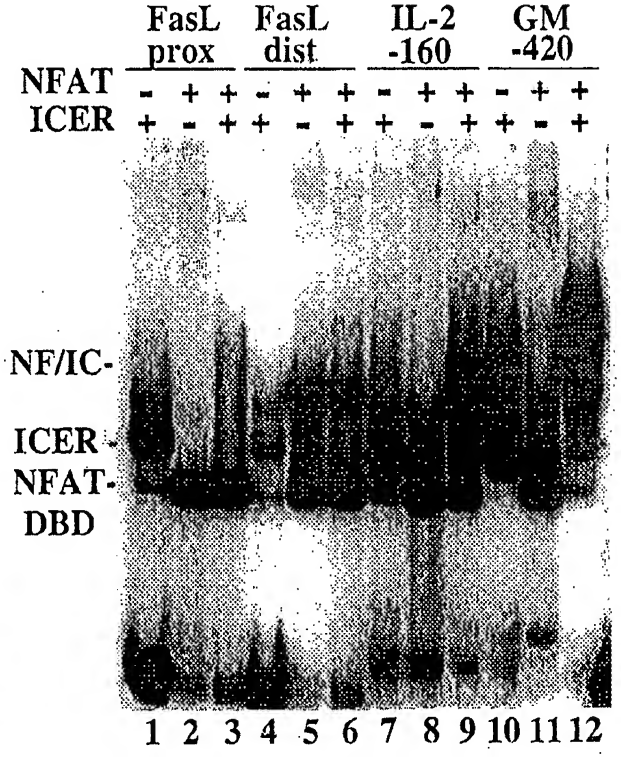

FIG.23C

NFAT

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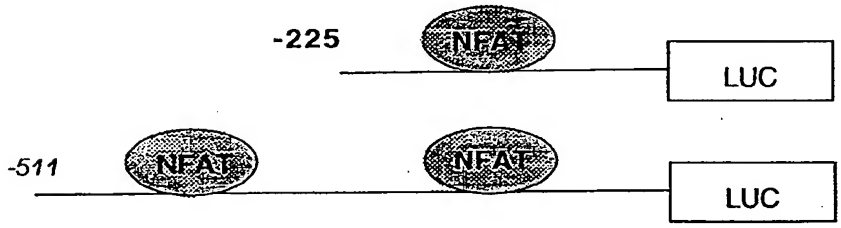

FIG. 24A

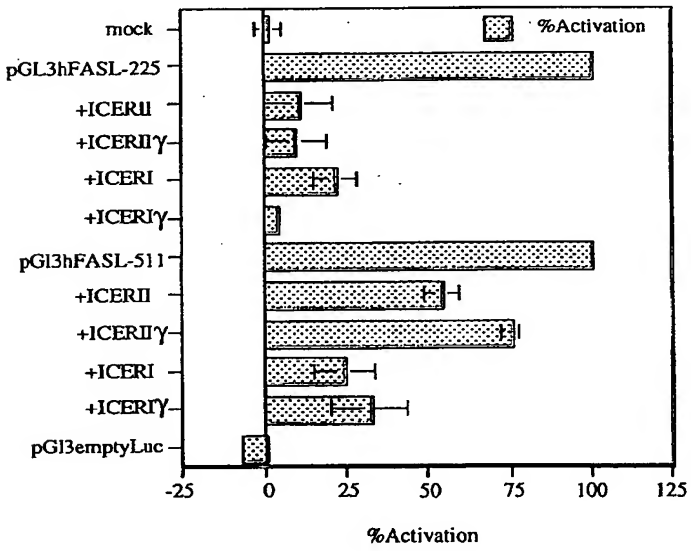

FIG. 24B

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Bodor, Josef
Weng, David E.
Koski, Gary K.
Czerniecki, Brian J.
Bodorova, Jana

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TO PREVENT ICER-MEDIATED INHIBITION OF IMMUNE CELL ACTIVITY

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                20                  25                  30

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Arg Ala Pro Thr Ala Ala Leu Pro Gln Gly Val Val Met Ala Ala Ser
            35                  40                  45

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Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Ala Leu
                100                 105                 110

aaa gat ctt tat tgc cat aaa gta gag taactgtctt tgacttggac            443
Lys Asp Leu Tyr Cys His Lys Val Glu
            115                 120

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gtttgtcaat agcatgcaaa aaagctttgt ttgccctttg cttctgcttt ttttcagg      681
gaa gct gcc aaa gaa tgt cga cgt cga aag aaa gaa tat gta aaa tgt      729
Glu Ala Ala Lys Glu Cys Arg Arg Arg Lys Lys Glu Tyr Val Lys Cys
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ctg gag agc cga gtt gca gtg ctg gaa gtc cag aac aag aag ctt ata      777
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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/00967

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C12N15/11 C12N9/00 A61K31/70
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BODOR J ET AL: "cAMP inducibility of transcriptional repressor ICER in developing and mature human T lymphocytes." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA., vol. 93, April 1996 (1996-04), pages 3536-3541, XP002105484 cited in the application the whole document --- -/--	1-42

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 1999	25/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 99/00967

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
A	LAMAS M ET AL: "Ectopic ICER expression in pituitary corticotroph AtT20 cells: effects on morphology, cell cycle, and hormonal production." MOLECULAR ENDOCRINOLOGY, (1997 SEP) 11 (10) 1425-34., XP002105485 cited in the application the whole document	1-42
A	FOULKES N S ET AL: "Transcriptional antagonist cAMP-responsive element modulator (CREM) down-regulates c-fos cAMP-induced expression." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1991 JUN 15) 88 (12) 5448-52., XP002105486 page 5451, left-hand column, line 34 - page 5452	1,2,4
A	FUJIMOTO T ET AL: "NOVEL ISOFORMS OF HUMAN CYCLIC AMP-RESPONSIVE ELEMENT MODULATOR (HCREM) MRNA1" JOURNAL OF BIOCHEMISTRY, vol. 115, no. 2, February 1994 (1994-02), pages 298-303, XP000676683 ISSN: 0021-924X cited in the application	
P,A	BODOR J ET AL: "Role of transcriptional repressor ICER in cyclic AMP-mediated attenuation of cytokine gene expression in human thymocytes." JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 APR 17) 273 (16) 9544-51., XP002105487 cited in the application see the whole article, and especially the conclusion.	1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/00967

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 20-24 (as far as in vivo methods are concerned) and claims 25-42 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.